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BOOK · JANUARY 2016

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Microalgae: Current Research and Applications

Maria-Nefeli Tsaloglou, Editor

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Chapter 1

Introduction to Microalgae

Carmelo R. Thomas (University of North Carolina Wilmington, USA)

Chapter 2
Cultivation of Marine Phytoplankton

Maria Jutson, Richard Pipe (The Marine Biological Association, UK) and
Carmelo R. Thomas (University of North Carolina Wilmington, USA)

The Cultivation of Marine Phytoplankton

Maria G.S. Jutson, Richard K. Pipe, Carmelo R. Tomas

1. Abstract

Please add 100-200 words describing the core concepts of the chapter.

2. Introduction

The isolation and cultivation of phytoplankton can be an essential, and also satisfying, process for studying microalgae in detail. Most species reproduce asexually, therefore establishment of unique strains in culture is relatively straightforward. Within the context of this chapter, the term strain will be used to define a genetically homogenous clone propagated from an individual microalgal cell. In essence, individual cells are isolated and placed in a suitable environment for growth. However, the actual process of establishing permanent cultures involves many steps and can entail prolonged periods of time. The validity of using cultured cells as a model for physiological function requires that culture conditions should resemble the natural environment as closely as possible.

Historically algal cultures have been grown on a small scale in research laboratories and universities for fundamental experiments and feeding other marine organisms. Most commercial growers have cultured a limited range of species principally as dietary supplements, for production of pigments or as food for aquaculture. Commercial production of algae is a small volume, high value market (Hennenberg et al., 2009) involving *Chlorella, Spirulina, Dunaliella, Haematococcus* and additional species for aquaculture (Meng et al., 2009).

Prior to delving into the details of cultivation a clear idea regarding the reasons for culturing microalgae needs to be established. To answer taxonomic questions, laboratory cultures may be essential for detailed morphological assessments. Other physiological questions on life cycle stages, including ‘cysts’, may require cultivated species. Detailed analyses of bioactive compounds, or pigments, also necessitate the production of a large biomass of the microalgal species from which these compounds can be isolated.

Microalgal cultures have many potential uses, including;

- Cell morphology
- Life cycle studies
- Physiological experiments
- Molecular genetics
- Aquaculture
- Studies of natural products and toxins
- Synthesis of organic compounds
- Production of nutraceutical and pharmaceuticals
- Carbon Dioxide Sequestration
- Biofuel production

3. Isolation of Culture Cells

3.1. Collection of Samples

For coastal and oceanic sites plankton is usually concentrated by gentle towing with a plankton-net, e.g. 10 minutes with a 200µm and a 600µm mesh net. Specialised water sampling bottles may be used for taking samples of known volume from a precise depth. Most water-sampling bottles consist of a cylindrical tube with stoppers at each end and a closing device activated by a messenger; they include

Kemmerer, Van Dorn, Niskin Bottles and Fjarlie bottles. Some microalgal species may also be collected from rock pools or the shoreline.

Having collected a phytoplankton sample, gentle handling and rapid transfer times are important factors as damaged and dying cells will not produce viable cultures. It is essential to make a record of relevant collection details. These could include; location, date, depth of sample, water temperature etc. When transporting collected samples, small plastic drinking water bottles are ideal. The bottles have to be rinsed with the sample water and filled so that no bubbles are present, this is particularly important for overnight shipping. The bottle may then be wrapped in wet towelling to insulate the sample. Ideally, a courier should be used as many can guarantee next day delivery. If the sample has been collected using a tow net or is from a very dense bloom, it has to be diluted prior to shipping. To dilute the sample, glass microfiber filtered (GF/F) water of the same salinity must be used. Shipping should be avoided during hot periods if possible or use cool packs separated from the sample with paper or towels. In general, unless the samples are of Arctic or Antarctic origin, ice should not be used during shipment.

3.2. Single-Cell Isolation

This is carried out using a micropipette and is probably the most common method for isolating individual phytoplankton cells. Traditionally a fine glass Pasteur pipette or a glass capillary tube has been used. A finely controlled picking out device, linked to a fine glass pipette, has been developed by Carmelo Tomas (pic) specifically for isolating individual cells. Alternatively a sterile extended fine tip plastic transfer pipette can be used. The aim of micropipette isolation is to pick up a single cell from the sample and deposit it, without damage, into a sterile droplet of medium. The sterile droplet containing the target cell, and probably other cells, is examined. This can be done using a multi-test slide (pic). Another useful method involves using a tissue culture inverted microscope with the aid of welled slides or welled plates. Slides can be purchased with cover glass-bottoms for critical observations and photographs, and these can be used with 40X and even 100 X objective lenses. When using any of these slides, pulled glass pipettes bent at 90° (pic) are useful. Standard upright microscopes may not be very comfortable because of the small working distance of the objective lens

and sample. Good quality dissecting microscopes are commonly used and can be very effective if the sample being selected is a larger one (>15µm). The down side of this type of microscope is the limited magnification (usually < 200 times total) and good lighting may be difficult to obtain. Washing and transferring cells from well to well in a microtiter plate or multi-test slide can be easily done. This process needs to be carried out enough times to ensure clean isolation of a single cell but without causing cell damage by excessive handling. Cells in specific plates can be observed day after day without disturbing the cells by simply placing the plate on the inverted microscope and observing them in a closed plate. Plates can be sealed with tape to reduce evaporation.

A newly isolated strain can be difficult to maintain so the optimum culture medium in which the alga will grow needs to be established. A reasonably reliable way of doing this is to simply put the isolates into several culture media and monitor growth. Knowledge about the growth medium used for closely related algae is often valuable. Daily microscopic examination should be carried out and once the culture is growing it can be transferred to a 100ml glass flask or plastic tissue culture vessel. Once the culture is established, it can be grown in larger volumes.

Other methods of isolation include; the use of agar, dilution techniques, centrifugation, flow cytometry and phototaxis (Andersen, 2005).

4. Sterilisation

4.1. Filtration

Filtration is mostly suitable for small volumes and heat labile components such as vitamins. Membrane filters with pore size of 0.2µm should remove contaminating organisms including bacteria; however viruses and fungal spores may still pass through. A 0.1µm filter can be used although filtration of large volumes will take a long time. A wide range of filter units and membranes are available including both autoclavable and single-use systems. Whilst filter sterilisation can be convenient it should be used with caution as filters used may leak compounds into the filtrate and absolute sterilisation is not guaranteed (Stokner et al., 1990).

4.2. **Autoclaving**

Autoclaving involves using high pressure (6.8kg) saturated steam at 121 °C to sterilise equipment and media. It is the standard microbiological system used as it guarantees sterility, including eradication of viruses and spores. Both large and small commercial autoclaves are generally used although for small volumes pressure cookers may also be suitable.

When autoclaving liquids it is essential that containers are only ¾ full, with screw caps partially loosened to prevent excess build-up of pressure. Under autoclave conditions the pH of seawater is raised as CO_2 is driven out of solution which may cause precipitation of salts. Precipitates should generally to be avoided as they tie up nutrients, metals and organics in a particulate form thus changing the availability of these components of the media. However, arguments have been put forward to suggest that precipitates are not problematic as they slowly release nutrients into the media over time and thus serve as a nutrient reservoir. To minimize the formation of precipitates in seawater or media, autoclaving may be carried out in Teflon bottles therefore avoiding adhesion of ions to the walls of the bottle. Precipitation may also be avoided by the addition of distilled water, usually ~5%, however salinity may be affected.

When autoclaving glass and plastic the high temperatures and pressure used will cause the release of substances from the vessel walls. Borosilicate glass such as Pyrex may release metals that have been used during manufacture. Polycarbonate bottles may continue to release plastic stabiliser for several months during the autoclave process. Some of these substances known as phthalate esters are toxic and can interfere with the growth of sensitive species. When autoclaving plasticware it is a good idea to have a small amount of deionised water in the container that will capture most of the dissolved plasticisers (phthalates). This can then be discarded under aseptic conditions just prior to filling the container with sterile medium. Autoclave tape is useful to indicate that the items have been treated and are sterile.

4.3. **Dry Heat**

Dry heat is generally carried out in a hot air oven and is useful for sterilising glassware (tubes, flasks, beakers etc) and glass pipettes. Glassware to be processed in this manner needs to be cleaned, dried and openings covered with aluminium foil to preserve sterility on removal from the oven. Items should be placed in an oven at ~160°C for a minimum of two hours. Pipettes stuffed with non-absorbent cotton can be placed in glass canisters or stainless dishes with lids although care must be taken not to heat for too long otherwise the cotton may burn.

4.4. Pasteurisation

Pasteurisation is a gentle and often effective method of sterilising seawater and media but is a time consuming process. It is particularly useful for sterilising liquids which should not be autoclaved or exposed to temperatures >100°C. In general, it involves heating a liquid to a specific temperature for a predefined length of time and then immediately cooling it after it is removed from the heat. For seawater and media heating to 80-90°C and maintaining at that temperature for an hour has shown to be effective. This process needs to be repeated a second time before the seawater or media are ready for use. Pasteurisation may be accomplished in a temperature regulated water bath with either a thermometer or thermistor to assess the temperature within the liquid. The advantages of pasteurisation are that it is gentle, normally destroys those organisms commonly found at lower temperatures and organic compounds remain intact. Delicate phytoplankton cultures normally respond favourably to pasteurised media. The disadvantages are that the processing time is normally longer than autoclave sterilisation and some organisms (viruses and some bacteria) may not be completely destroyed.

4.5. Ultraviolet Treatment

Ultraviolet treatment is not generally used for the preparation of culture media as it is not considered a sufficiently reliable technique for sterilisation. UV light does not penetrate water uniformly and microbes may attach to vessel surfaces potentially rendering sterilisation ineffective. For ultraviolet treatment to be effective, the UV light should be of sufficient strength as bulb strength varies with

time. The water should be kept in quartz glass tubing which is transparent to UV light and maintained in the UV light stream for the required time to destroy all microbes.

4.6. **Microwaving**

Microwaving has the advantage of speed for sterilising small volumes (1-1½ litres) where it has been shown to kill bacteria and fungi within 10 minutes (Keller et al.1988). However, as the power, and efficiency, of microwave ovens varies a great deal each unit will require protocols to be developed. In general this method is not practical for large volumes but is useful for laboratories that do not have autoclaves.

5. Culture Maintenance Conditions

5.1. Temperature

Ideally, temperature should reflect the conditions from which the algae were isolated. Therefore; polar isolates should be maintained at <10°C, temperate isolates at 10-25°C and tropical isolates at >20°C. Temperatures above 30°C are usually fatal for marine algae. Temperature stability should be kept to ± 2°C, where possible. Incubation temperatures higher than 20°C may be combined with increased light intensities to prevent photo-inhibition or damage.

5.2. Lighting

Traditionally, cultures are housed in a constant temperature room with a large north (in the northern hemisphere) facing window, usually with supplementary lighting. However, very few facilities have the luxury of natural lighting and most have to rely on indoor lighting provided by various lighting systems. One most commonly used system employs cool white fluorescent bulbs to give the adequate flux density to grow cells properly. Various bulbs are available, some with spectra designed for terrestrial plants; however, these are expensive and give no advantage over the plain cool white bulbs. A recent innovation is the advent of light emitting diodes (LED) that now come in a variety of power strengths and spectra, similar to those of other sources. These bulbs are presently more expensive than the fluorescent ones but consume a fraction of the electricity and normally have a 50,000 hour burn resulting in a significant savings over more conventional bulbs. When using fluorescent tubes they should be placed at a distance from the cultures, so as not to cause any overheating of the algae. Photo periods are required for the maintenance of most algae. Continuous light can be detrimental and may kill some algae. In most culture collections the light/dark regimes vary between 12:12 and 16:8 hour light/dark. The regime used will depend on whether the cultures are kept in a room where natural light is available or not and, if so, the time of year. Direct sunlight should always be avoided. Over-illumination is a widespread mistake in the maintenance of cultures. Problems include; localised heating of algae, photo-oxidative stress and bleaching (loss of pigmentation) effects.

5.3. **Culture Media**

Media for the culture of marine phytoplankton in the laboratory can be either defined or undefined. Defined media use high quality deionised water to which salts are added, together with the nutrients required to make the formulation of choice. They are generally preferred by physiologists who control all variables as closely as possible. The general finding is that defined media will not grow all species and sometimes result in species growing abnormally. Therefore, defined media are generally best used for specific needs but not for maintaining cultures for long periods of time.

Undefined media consists of a seawater base, either natural or artificial, which is then supplemented with additional nutrients. The media is best prepared using good quality, oligotrophic seawater collected some distance from land to avoid runoff contamination. Collection of seawater needs to be done carefully with pumps and tubes that are non-metallic so as to avoid contamination. The quality of the seawater may be improved by ageing for a few months to allow for bacterial degradation of inhibitory substances. It is usually kept in seawater-conditioned polyethylene carboys or tanks and stored in a cool, dark place to discourage growth of photosynthetic organisms. Proprietary brands of artificial seawater may be used if natural seawater is not readily available. Seawater must be filtered prior to use to remove particulate matter. Pre-filtering (e.g. Whatman no. 1) can be performed to remove larger particles before further filtration through 0.45 µm filter.

The formulation of each medium will have its own specific nutrient composition. The macro nutrients, nitrogen and phosphorous, are major additional components of all culture media recipes. Nitrate is the preferred form of additional nitrogen, although a few recipes use ammonium. Organic nutrients are another consideration and for nitrogen, a variety of amino acids have been used. Inorganic orthophosphate is the usual form of additional phosphorous, although organic glycerophosphate can be used. Some algal groups including diatoms, silicoflagellates and some chrysophytes also have a requirement for silica in the culture medium. Recipes vary but these macro nutrients are essentially the same for different media for example the F series (f/2, K, L1 etc). The components which do vary in the different media are the trace metals and perhaps particular vitamins. Low concentrations of some trace metals are essential for algal growth. These trace metals include;

iron, zinc, copper, cobalt, manganese and nickel. Vitamins are routinely added to nearly all formulations and consist of a combination of thiamin (B_1), biotin (H) and cobalamin or cyanocobalamin (B_{12}). Concentrations of trace metals and vitamins vary between different media recipes but are usually in the range 10^{-7}–10^{-12} moles. These components will usually need to be made up in two stages. Primary stocks are made up which are then diluted into a working or secondary stock. These primary stocks are long lived and can be kept frozen at -20 °C for extended periods of time.

There are several nutrients of particular note worth mentioning that have special usages. One of these is the micronutrient selenium. Both K and L1 medium have this as an important component. Although the exact physiological function of selenium is not well understood, it was not until the inclusion of this metal in media that hard to grow benthic or open ocean species were successfully cultured. The form of selenium is important and should be the reduced form. The common reagent used is selenous acid (H_2SeO_3) and is used at 10^{-8} molar concentration. Iron is another critical element that offers some difficulty in preparation. For many years the inorganic form of iron as ferric chloride ($FeCl_3.6H_2O$) was used but this may precipitate within a short time, leaving the amount of iron in solution unknown. Even in a precipitated solution, the amount of iron added in the specified volume is generally considered to be far beyond the requirement. However, an alternative to ferric chloride is a chelated form of iron as sequestrene (di-ferric sodium EDTA salt). This form of iron is easily soluble, remains in solution longer than ferric chloride and is commonly used as a higher plant supplement. Historically, the use of soil enrichment extract was found to be essential for the growth of many algal cultures (Pringsheim, 1946). Soil extract enriched medium is still used successfully by some culture collections today. The most suitable type of soil to use is sieved sandy loam which is lime free and has not been treated with any fertilisers and pesticides.

The preparation of different culture media varies considerably, however the end results need to be sterile. Sterilisation can be of each component separately or alternatively the complete medium once prepared. There are many methods utilised to accomplish sterile culture media including; filtration, autoclaving, pasteurisation and ultraviolet treatment.

5.4. **pH**

The control of pH in culture media is important as certain algae will only grow within narrowly defined pH ranges. The pH of natural seawater is usually around 8. Because of the natural buffering capacity of seawater it is quite easy to maintain the pH of marine culture media. The buffer system may be overwhelmed during autoclaving, when high temperatures drive CO_2 out of solution and hence cause a shift in the bicarbonate buffer system and thus an increase in pH. As culture medium cools after autoclaving, atmospheric CO_2 enters the solution but if normal pH is not fully restored the level will need to be adjusted. Alternatively, the pH of seawater may be lowered prior to autoclaving (adjustment to pH 7-7.5 with 1M HCl) to compensate for subsequent increases.

Some media recipes include the addition of extra buffer, either as bicarbonate, Tris-hydroxymethyl-aminomethane (Tris), or glycylglycine to supplement the natural buffering system. Glycylglycine is rapidly metabolised by bacteria and hence should only be used with axenic cultures. These additions are generally not necessary if media are filter sterilised, unless very high cell densities are expected.

Very dense cultures of microalgae may take up sufficient CO_2 to also raise pH level. The problem of CO_2 depletion in dense cultures may be reduced by having a large surface area of media exposed to the atmosphere relative to the volume of the culture, or by bubbling with either air (CO_2 concentration c.0.03%) or air with increased CO_2 concentrations (0.5 to 5%). Unless there is a large amount of biomass taking up the CO_2, the higher concentrations could actually cause a significant decline in pH levels. When bubbling is employed, the gas must first pass through an in-line 0.2µm filter unit to maintain sterile conditions.

5.5. **Aeration**

For many microalgal species, aeration is not an option since the physical disturbance may inhibit growth or kill cells. However, it may be necessary when cells must be kept in suspension in order to grow. This is particularly important for heterotrophic dinoflagellates. In concentrated cultures aeration may also be necessary to prevent nutrient limitation effects due to the stacking of cells and to increase gas diffusion. It should be noted that in the ocean cells seldom experience turbulence, hence

mixing should be gentle. Aeration methods include; bubbling with air, gentle manual swirling, using a plankton wheel or roller table. Most cultures do well without aeration, particularly when not too concentrated, but when possible gentle manual swirling, once a day, is recommended.

5.6. **Culture Vessels**

Containers for culturing algae should be non-toxic, enable illumination and be capable of withstanding sterilisation. Borosilicate glass has been used traditionally but is increasingly being replaced by plasticware as predicted by Pringsheim back in 1946. Glass vessels are relatively inexpensive but are labour intensive to maintain as they require cleaning and sterilising. Pre-sterilised, disposable, plasticware can be purchased in ready-to-use packages but is relatively expensive, both economically and environmentally. Plastics can be coated with substances which may enhance growth of many algae. A range of plastics may be suitable for culturing including; Teflon, polycarbonate, polystyrene, polyethylene terephthalate (PET) and polyethylene terephthalate copolyester glycol modified (PETG).

Plastic vessels are available in a wide range of formats from 96 well microtiter plates to large volume carboys. Most tissue culture flasks are flat enabling cultures to be grown on shelves. The caps may be solid or can have a porous insert to allow gaseous exchange whilst maintaining sterility. The flaks with porous inserts often give better results for some species. Most sterile plasticware is sterilised by gamma radiation which does not inhibit growth in the majority of species. Some flasks used in medical procedures are gas sterilised. These should be rinsed with sterile deionised water prior to use in order to remove any traces of the sterilising gas.

Erlenmeyer flasks have traditionally been used for maintaining laboratory cultures. Whichever container is chosen it should enable gaseous exchange but prevent contamination and limit evaporation. Various closures may be utilised including; cotton wool, metal or glass lids, screw caps, foam bungs and BugStoppers. Cotton wool plugs should be covered with an aluminium cap or grease-proof paper fixed with a rubber band to reduce evaporation.

6. Culture Methods

6.1. Batch Culture

An algal inoculum is placed in a fixed amount of complete culture medium and incubated in a favourable environment for growth. Resources are finite when growing algal cultures in a limited volume. When the resources present in the culture medium are abundant, growth occurs according to a sigmoid curve, but once the resources have been utilised by the cells the cultures die, unless supplied with new medium. In practise, this is done by routine sub-culturing, i.e. aseptically transferring a small volume of existing culture in late log/stationary phase to a large volume of fresh culture medium at regular intervals. The characteristic pattern of growth involves; *lag phase* where cells are adapting to their environment and do not divide; *exponential (log) phase* where cells multiply rapidly; *stationary phase* where the number cell division is limited by the reduced nutrients and as a result the rate of cell growth matches the rate of cell death and *death phase* where cells run out of nutrients completely and die.

When sub-culturing aseptic technique should always be adopted and where possible a lamina flow hood used to reduce any possibility of contamination. For routine maintenance, the goal is to sub-culture the alga at the end of its exponential growth phase. This depends on the sensitivity of the strain, with sensitive strains requiring a transfer cycle of 1-2 weeks and the most robust strains only requiring transfer every 2-3 months. At the Plymouth Collection, the majority of strains are sub-cultured every 4 weeks. The volume of inoculum is dependent upon cell density but is usually in the range of 1-5% of the fresh culture medium. Algae kept on agar slopes can be transferred once every 6 months.

6.2. Continuous Culture

Resources are potentially infinite in continuous cultures. Therefore, the algae are maintained at a chosen point on the growth curve by the regulated addition of fresh culture medium. In practise, a volume of fresh culture medium is added automatically at a rate proportional to the growth rate of the

alga, while an equal volume of culture is removed. By doing this the growth limiting substrate is replenished and any growth inhibitory products are removed.

6.3. **Semi-continuous Culture**

Cells in an actively dividing state are maintained in culture by periodically draining off a proportion of the depleted medium and replenishing it with fresh medium. This system is not sustainable indefinitely as the build-up of inhibitory or toxic by-products will ultimately result in cell death.

6.4. **Culture of Large Volumes**

Scaling up of algal cultivation has been carried out in both open-air systems or in specially-developed photo-bioreactors. Commercial cultivation of microalgae is limited to a small number of species and is currently carried out, almost exclusively, in open systems. This is because large ponds and raceways are relatively cheap to construct and operate compared with photo-bioreactors. The majority of commercial open-air systems are sited in tropical or sub-tropical regions where there is less fluctuation in light and temperature. The main disadvantages of these systems are; the risk of contamination by predators or other algae, evaporation, the footprint required and relatively low cell densities obtained. A very large number of closed photo-bioreactor systems have been developed. The main benefits of a closed system are; full environmental control, risk of contamination is greatly reduced and a much smaller footprint than open systems. The major disadvantages are expense of construction and running costs.

6.5. **Harvesting Cultures**

In order to remove large quantities of water and process large algal biomass volumes several steps are required including; separating algae from the medium, drying the cells and processing to obtain the desired product. Harvesting methods depend primarily on the type of algae being grown. Common harvesting processes involve flocculation, centrifugation and filtration. Probably the most popular method of harvesting large quantities of culture is to use continuous flow centrifugation. These units are made to run continuously and result in a moist paste that can be dried further or stored in cool or frozen conditions. A 60 liter bag culture can be harvested in less than 2 hours. All methods should be

energy-efficient and relatively inexpensive so selecting easy-to harvest algae is important. Methods for drying and further processing depend upon the product required.

7. **Contaminating Organisms**

Microalgal cultures are susceptible to a wide range of contaminating microorganisms including; viruses, bacteria, fungi and protozoa. Cultures may be grown either axenically i.e. not contaminated by or associated with other living organisms or xenically i.e. contaminated by or associated with other living organisms. Within the context of algal culturing these terms usually refer to bacteria. The interactions between bacteria and phytoplankton species are complex (Doucette, et al., 1998). When growing microalgae in culture it has been shown that some individual strains of bacteria may inhibit growth whilst others act as a stimulant (Biddanda et al., 2006). However, it is generally agreed that the performance of many algal cultures is better with some bacterial contamination as some microalgal cultures may die when made axenic. This is probably due to the termination of obligate symbiotic relationships with bacteria.

If a biological contaminant appears in a culture, a clean clonal culture may be re-established by isolating a single cell from the contaminated culture, using a micropipette. Alternatively, the culture can be streaked on an agar plate in the hope of attaining a colony free of contaminants. Neither of these methods work particularly well for eliminating bacteria attached to the surface of the algal cells. However, placing a test tube of microalgal culture in a low-intensity 90 kilocycles/sec ultrasonic water bath for varying lengths of time (a few seconds to tens of minutes) can sometimes physically separate bacteria without killing the algae, making it easier to obtain an axenic culture by micropipette isolation.

In order to achieve an axenic culture it is often necessary to use antibiotics. One of the most successful methods involves using an actively growing culture of algae and exposing it to a mixture of penicillin, streptomycin and gentamycin for around 24 hours. This drastically reduces the growth of bacteria whilst allowing the microalgae to continue to grow. The chances of obtaining an axenic culture when using micropipette or agar streaking isolation methods are then greatly increased.

Different algal species tolerate different concentrations of antibiotics, so a range of concentrations should be used (generally 50-500 mg/l). Other antibiotics that can be used include; chloramphenicol, tetracycline and bacitracin. Antibiotic solutions should be made with distilled water and filter-sterilised (0.2µm filter units) into sterile tubes and may be stored frozen until use. Another approach is to add a range of antibiotic concentrations to a number of subcultures and then select the culture that has surviving algal cells but no surviving bacteria or other contaminants.

Sterility of cultures can be checked by microscopic examination, either with or without a fluorescent dye such as DAPI. Alternatively a small amount of sterile bacterial culture medium (eg. 0.1% peptone) can be added to a microalgal culture and observed regularly for bacterial growth. Absence of bacterial growth does not, however, ensure that the microalgal culture is axenic, since the majority of bacteria do not respond to standard enrichments. In reality there is no way of demonstrating that a microalgal culture is completely axenic. In practise, therefore, axenic usually means 'without demonstrable unwanted prokaryotes or eukaryotes'.

Fungal contamination is generally worse than bacterial contamination because fungi are harder to eliminate by either physical or chemical methods. If fungal contamination is suspected it can be checked for using peptone or malt extract dissolved in the algal culture medium. Current methods for controlling fungal contamination rely on using aseptic techniques when handling cultures. If cultures become contaminated, physical separation of algal cells from fungal cells, on agar plates may be carried out using a microscope. Some algal strains show positive phototaxis and this ability may also be used to physically clean cultures. A potentially convenient and effective control measure is to add a fungicide to solid media to either prevent fungal contamination or to clean up contaminated cultures. Fungicides which have been used successfully include; carbendazim, thiophanate-methyl, and benomyl, (Mahan et al., 2005). These fungicides are considered broad-spectrum fungicides of the benzimidazole family. Cultures may be cleaned by streaking the contaminated algae onto agar plates containing 40 µg/mL of the fungicide. The algae can be re-streaked onto fresh agar after 5–7 days of growth. This process may need to be repeated several times for heavily contaminated cultures.

Contamination of laboratory cultures with protozoa is not usually a problem when aseptic techniques are employed and cultures can be re-isolated relatively easily. Whereas problems may arise in cultures containing unwanted microalgae such as diatoms which grow very quickly and can often outcompete other algae. Diatoms can be eliminated with the addition of germanium oxide to the culture (Lewin, 1966)

8. **Cell Counting**

In order to determine the concentration of cells in a culture it is necessary to count the cells in a given volume. There are several methods available for counting algal cells. These may direct or indirect and range from simple microscope counting chambers to sophisticated electronic devices. The most accurate is to actually see and count cells with a microscope and various chambers that make the counting volume accurate for extrapolation to larger volumes. However, the choice of equipment depends on several factors, including; culture density, size and shape of the cells and presence of extracellular material such as mucilage. While the indirect methods may seem less tedious, in that a surrogate is used for biomass (chlorophyll, fluorescence, optical density, electronic particle counting) the major drawback is that these methods need to be carefully calibrated against standards with cell counts. In addition, by using relative fluorescence or particle density, there is no assurance that only cells are being counted or measured. These counted samples should at least occasionally be verified by direct observation. Variables including, chlorophyll per cell, detrital load and coincidence in particle counting should be of concern when using indirect methods.

8.1. **Sedgwick-Rafter Counting Cell**

The chamber is rectangular (50x20 mm), 1 mm deep, with an area of 1000 mm^2 and a volume of 1.0 ml. It is best used for relatively large cells, 20-500 µm with cell densities ranging from ~30 per ml up to about 10^5 per ml. The base is divided into 1 mm squares. A cover glass traps liquid to the correct depth. The chamber must be completely filled with no air bubbles present. Using an inverted microscope or low power compound microscope, cells in each cubic millimetre can be counted. Where cells lie on the lines, only count those organisms on two sides of the square. The chamber and

cover-slip must be perfectly clean; this is usually achieved by rinsing in ethanol or methanol. Fixation is necessary for motile cells; this is commonly done using Lugol's iodine or alternatively a formaldehyde based fixative. For Lugol's iodine, mix together 10g potassium iodide, 5g crystalline iodine and 100ml distilled water. Once dissolved add 1 drop (~0.05ml) per 5ml of algal sample.

8.2. **Haemocytometer**

The haemocytometer has 2 chambers each with nine 1mm squares with several layers of subdivision. One of the most common rulings is the 'Improved Neubauer' which comprises a grid 3mm x 3mm, divided into nine $1mm^2$ areas, with a depth of 0.1mm. Another useful chamber is the Fuchs-Rosenthal, which also has a total ruled area of $9mm^2$ but has a depth of 0.2mm, making it useful for bigger cells. However, even with a depth of 0.2mm, the size of cells which can be counted accurately is limited to around 70µm, as larger cells will seldom form an even distribution in the chamber. Motile cells will need to be fixed when using this method. The chamber and cover-slip must be perfectly clean; this is usually achieved by rinsing in ethanol or methanol. The cover-slip is firmly fixed to the chamber by cohesion as shown by the presence of diffraction rings. It is essential that the cover-slip is symmetrically placed over the double rulings to prevent non-random cell distribution. The cell suspension is transferred to the chamber using a fine pipette. The chamber must be filled using a single application, without overflowing into the channels and must not contain bubbles. The cells must be allowed to settle prior to counting. For cells touching the centre line of the triple ruling it is important that only 2 sides of the square are included in the count and cells touching the centre line of the other 2 sides are excluded. To maintain an acceptable degree of error, at least 50 cells should be counted. To calculate cell density:

Cells per µl = number of cells counted x 1/area x 1/depth x dilution

Other haemocytometer rulings are available including; Thoma, Burker, Malassez, Nageotte and Petroff-Hausser.

8.3. **Palmer-Maloney Slide**

The counting chamber has no rulings, is circular, 17.9 mm in diameter x 0.4 mm deep, with a volume of 0.1 ml. The chamber is provided with two charging channels, 2 mm wide x 5 mm long. To use the Palmer-Maloney counting chamber, a cover-slip is placed on the edge of the chamber and the sample introduced, as the sample fills the chamber, it draws the cover-slip over the chamber. The Palmer Maloney is useful for organisms with a size range of 5–150 μm.

8.4. **Secchi Disc**

A Secchi disc is a standard disc, either plain white or with alternating black and white quadrants, which is lowered into the sample until it is no longer visible. This depth of disappearance, called the Secchi depth, is a measure of the turbidity of the water. The Secchi depth is proportional to the algal cell density. Once calibrated using direct cell counting it can be useful for estimating growth of a microalgal culture. The Secchi disk readings can only provide an estimate of turbidity, because errors can arise due to factors like lighting conditions or individual perception. However, it is an inexpensive and straightforward method of measuring water clarity and thus estimating cell density. Because of the potential for variation between operators methods should be standardised as far as possible.

8.5. **Spectrophotometer**

The use of a spectrophotometer to measure cell concentration provides an indirect method that correlates the algal density with light absorbance at specific wavelengths. Spectrophotometers can use either cuvettes or microplates which enable cell density to be measured in a wide range of volumes. The method can be reliable and also easy to setup for automatic monitoring systems (Rodrigues et al., 2011). The drawback is the limited accuracy because the cells are not counted directly as the machine measures light, not cells. This, combined with the stochastic nature of liquid cultures, enables only an estimation of cell numbers. The spectrophotometer can also be used to measure the chlorophyll concentration in cultures. Chlorophyll A constitutes approximately 1-2% (dry weight) of algal biomass which makes it a convenient indicator of algal biomass. However, the chlorophyll a content

in an algal cell is not constant and varies with the nutritional state of the cell and this will obviously affect the accuracy of the cell density estimates derived.

8.6. **Coulter Counter**

The machine has one or more micro-channels separating two chambers containing electrolyte solutions. As culture medium containing the algal cells passes through the micro-channel, each algal cell causes a brief change to the electrical resistance of the liquid. The counter detects these changes in electrical resistance and thereby counts each cell. The advantages of using a Coulter Counter are the accuracy it provides and the relative simplicity, enabling large numbers of samples to be counted efficiently. The disadvantages are that it does not discriminate between algal cells and other particles, dense culture needs to be diluted to get an accurate count and they are relatively expensive.

Flow cytometer

The most sophisticated and expensive method for counting cells is to use a flow cytometer. The culture containing the algal cells flows in a narrow stream in front of a laser beam and a light sensor detects the light reflected from the cells as they hit the beam. Flow cytometers can also analyse the shape of cells and their internal and external structures as well as measuring specific components of the cells. The expense and complexity of flow cytometers means that they are rarely used for the sole purpose of counting cells. Flow cytometers incorporating a cell sorter may also be useful for making rapid single cell isolations.

9. **Cryopreservation**

The purpose of cryopreservation is to maintain cells at extremely low temperatures whilst maintaining viability. The majority of cryopreservation protocols for microalgae use a 2-step controlled rate freezing methodology with the aid of cryoprotectants which are used to minimise damage to the cells. Commonly used cryoprotectants include; dimethyl sulphoxide (DMSO), glycerol and methanol. There are two components of freezing injury; direct damage from ice crystal formation and secondary damage caused by the increase in concentration of solutes as progressively more ice is formed. Lethal intracellular freezing can be avoided if cooling is slow enough to permit sufficient water to leave the

cell during progressive freezing of the extracellular fluid. The rate of freezing varies according to the size of the cells and their water permeability.

A general protocol for microalgae involves cooling the cultures at a rate of -1°C per minute to a holding temperature of around -40°C. The rate of cooling can be controlled using either a programmable freezer or a small portable freezing unit such as a “Mr Frosty”. The samples are then plunged into liquid nitrogen at -196°C and transferred to long term storage. This can be in liquid nitrogen (-196°C), the cold vapour phase above liquid nitrogen (-165°C) or an ultra cold freezer (-150°C). Storage temperature must not exceed -130°C as this is the threshold for ice re-crystallisation, (Mazur, 1966). Cryovials containing specimens should not be left out of the freezer for longer than 3 minutes to ensure that they do not rise above -130°C.

There are several protocols available for the regeneration of frozen cultures. They all involve removal of the sample from the storage container and rapid transfer into a water-bath set at 37-40°C. The cryovial is gently agitated during thawing until all ice has just melted; this usually takes less than 2 minutes. The microalgae can then be directly transferred into a flask containing fresh growth medium. Alternatively, the sample may be gently centrifuged and the pellet re-suspended in fresh culture medium. This process is repeated before the culture is transferred to a larger volume of medium. Before returning to normal culturing conditions the sample can be kept in the dark for 24 hours and then in reduced lighting for a further 24-96 hour. This may be important to ensure that cells are not subjected to light levels likely to induce photo-oxidative stress during the recovery phase.

The advantages of cryopreservation of microalgae include; effectively guarantees phenotypic and genotypic stability, can protect culture against microbial contamination and potential errors arising from handling and labelling during multiple sub-culturing, requires minimal comparative storage space and may reduce the cost of long-term maintenance. The disadvantages include; initial set-up costs are expensive, storage equipment needs a constant supply of liquid nitrogen, back-up facility is required in case of mechanical failure, cultures are not instantly available as post-thaw regeneration times are relatively long.

It must be stressed that there is no universal method for the successful preservation of all microalgal species currently in culture. Different taxonomic groups, and even strains within a species, can exhibit differences in response to the stresses imposed by culture preservation and resuscitation. In general, small easy to culture microalgae tend to be amenable to cryopreservation whereas larger more sensitive species can be problematic.

The ability to cryopreserve samples can be considered as a prerequisite for a culture to be called a "strain". However, as currently only a fraction of algal species are successfully cryopreserved this has not been generally adopted by the phycological community.

10. **Record Keeping**

When isolating algae, it is essential to keep full records of any strains retained in culture. These should include; an identification number, the name of the species (if known), the collection date, the source of the material, details of the collection site, the method of isolation, the name of the isolator, the culture medium used and any other useful information or special requirements. When maintaining a large number of cultures it is advisable to store this information in a suitable database with a back-up facility.

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Chapter 3

Gene Expression in Phytoplankton

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PHYTOPLANKTON GENE EXPRESSION

John H. Paul

1. Abstract

Phytoplankton photosynthesis accounts for 50% of the primary production on our planet and forms the base of marine food webs. Phytoplankton can form toxic blooms, take up and release greenhouse gasses, and be responsible for fueling the biological pump. The availability of genomes of several marine phytoplankton including diatoms and EST libraries of several dinoflagellates have opened the door for understanding gene expression and regulation in this globally important group of photoautotrophs. This

review documents early work in phytoplankton gene expression through current studies in marine phytoplanktonic transcriptomes and proteomes. All informatics analyses are hampered by the lack of reliable gene annotations. Progress is being made in gene function identification through the use of endogenous gene silencing mechanisms. Understanding the control of gene networks is in its infancy but promises to be a fertile area of research for the next generation of phytoplankton ocean scientists.

2. Introduction

A major goal amongst microbial ocean scientists is to understand the *function* of microbial populations in marine environments and how these respond to perturbations on both short and long time scales. There is a real need to understand gene function, not only in marine phytoplankton, but in every biological system on the planet. But what is meant by "gene function"? Tian et al. (2011) points out that the meaning of gene function might depend on one's frame of reference (Tian (2011). A biochemist might describe a gene function in strictly catalytic terms: The enzyme oxidizes a carbonyl group to an organic acid. To a cell physiologist, the participation in a pathway is the gene function. To a clinical researcher, the change in pathogenicity of the microbe caused by gene expression will be important. Thus, every notion of "gene function" inherently has 3 aspects: molecular function, biological process, and cellular component. Tian et al. pointed out to understand all the actions resulting from gene expression an ontology system covering all major aspects of gene activation was needed. The Gene Ontology (GO) system provides this activity, therefor being better for annotation (Tian et al., 2011).

The GO database is a relational database comprising the GO ontologies and the annotations of genes and gene products to terms in the GO. The advantage of housing both the ontologies and annotations in a single database is that powerful queries can be performed over annotations using the ontology.

However we know now the genome centric world is a simplistic view of the biosphere because external genomes can modify gene expression through certain types of symbiotic parasitism. In other situations organisms have outsourced control of their genomes to other microbes in their environment via the

process of epigenetics. Temperate and latent viruses are now known to control aspects of the host's gene expression through the action of repressors and transcriptional regulators.

In this review I discuss the evolution of methods and approaches to the measurement of phytoplankton gene expression. I then address how these approaches are used to measure the regulation of phytoplankton genes involved in C, N, P, and Fe metabolism in the ocean.

2.1. The Approaches to the Measurement of Gene Expression in the Oceans

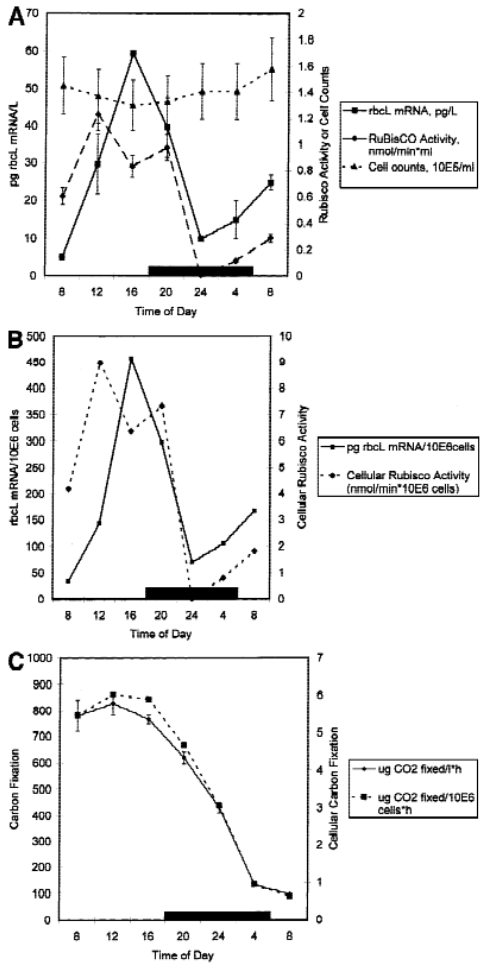
In a larger sense, gene expression means the detection of the activities encoded in the DNA, or those encoded by the genes identified (open reading frames or ORFs). More narrowly, gene expression refers to the *activation* of the gene from dormant (un-transcribed) to the actively transcribed state (ie. production of a sense transcript). Other notions of gene expression limit the meaning to production of the protein encoded by the ORF in question, while still other concepts of expression are limited to the production of an *active* protein or enzyme. Thus gene expression can encompass transcription, translation, and post-translational processes.

Early gene expression research relied upon detection and sometimes quantification of the target gene mRNA in what was called "Northern hybridization" (Kinoshita et al., 1998; Lin and Carpenter, 1998; Pichard, 1993). By this approach, RNA was blotted on charged nylon membranes and hybridized to radiolabeled probes (Paul, 2001). The blots included standards and if these were quantitated one could get accurate concentrations of similar mRNAs in the oceans (Pichard, 1993; Pichard et al., 1996). To verify that RNA and not DNA was being detected, blots were probed with labelled antisense probes to detect transcripts while identical blots were probed with labelled sense probes to correct for non-specific hybridization or contamination of the sample with target DNA (Pichard, 1991). Quantification was accomplished by use of a molecular imager (Paul, 2001). This approach was tedious, usually requiring radioisotopes and production of run-off transcripts and a myriad of other protocols.

An extension of the blot and probe approach to detecting and quantitating gene expression is the microarray approach (Tiquia, 2004). Ward (2008) developed a “phytoarray” that contained oligonucleotides targeted for phytoplanktonic carbon fixation and assimilatory nitrate reduction. This approach successfully detected the shift from diatom C and N assimilation in the early spring bloom to that of haptophytes and flagellates later in the summer (Ward, 2008).

Reverse transcription PCR (RT-PCR) was a sensitive method to detect rare transcripts for which nominal sequence information was available. Le Blanc et al.,(1999) developed a semi-quantitative RT-PCR assay encoding a fucoxanthin, chlorophyll a/c-binding protein (FCP) in the centric planktonic diatom *Thalassiosira weissflogii*. With the development of Real time RT-PCR (RT qPCR), detection and quantification of rare transcripts of natural populations has now become a trivial undertaking (Leblanc, 1999). Wawrik et al. (2002) developed a RT qPCR assay for quantitating diatom and pelagophyte ribulose bisphosphate carboxylase/oxygenase large subunit (*rbcL*) transcripts. Although the goal of this research was to design a qPCR assay specific for diatoms only, the pelagophytes could not be resolved from the diatoms based upon rbcL sequence data (Wawrik et al., 2002). The desire to have RT qPCR assays for specific ecotypes is often confounded by the reality of sequence conservation or diversity amongst the ecotype groups. For example, although we could easily design a RT qPCR assay for high light *Prochlorococcus*, the low light clade *rbcL* was far too diverse to design a qPCR assay (John et al., 2009). Instead we developed RT qPCR assays for haptophytes (many golden flagellates), Form 1A *rbcL* of *Synechococcus*, diatoms/pelagophytes, and high light *Prochlorococcus* (John et al., 2012; John et al., 2009). Non-specific amplification strategies have been developed to neutralize some of the selectivity of traditional qPCR approaches. mRNA arbitrarily primed reverse transcription-polymerase chain reaction (RAP RT-PCR) is a method to capture unknown mRNAs resulting from a manipulation or a disturbance (Kinoshita et al., 1998). Usually one primer is polydT that initiates cDNA formation of the eukaryotic mRNA pool. Arbitrary primers are then used in the PCR to produce a family of DNAs separated on a 2D gel. For cultures this method yields reproducible displays of control and perturbed conditions.

As mentioned above, transcription does not equal gene expression, even though many biological processes are transcriptionally regulated. A more stringent interpretation of the definition of gene expression is production of the enzyme or structural proteins encoded by that gene. Vergara and co-workers (1998) studied nitrate reductase (NR) expression in the diatom *Thalassiosira weissflogii* (Gru.) Fryxell et Hask. Here they developed antibodies toward the NR as well as detecting NR activity as the penultimate measure of gene expression (Vergara et al., 1998).



Similarly, Paul et al. (2000) measured *rbcL* mRNA, RUBISCO enzyme content, and whole cell carbon fixation in both a marine prymnesiophyte and a marine *Synechococcus* over diel cycles and found all three measurements to co-vary, indicating that transcript abundance was a good proxy for gene expression in this system.

Figure 1. Diel studies of the regulation of carbon fixation in *Pavlova gyrans.* Panel A: *rbcL* transcript abundance, and RUBISCO enzyme activity, and cell abundance over time. Panel B: Cellular levels of *rbcL* transcript abundance and RUBISCO enzyme activity. Panel C: Photosynthetic carbon fixation rates (Paul et al., 2000)

Long serial analysis of gene expression (SAGE) has been used to identify *E. huxleyi* genes responsive to nitrogen (N) or phosphorus (P) starvation (Dyhrman et al., 2012).

2.2. The Genomic Approach to Understanding Gene Expression in Phytoplankton

As the most successful group of eukaryotic phytoplankton in the modern ocean, diatoms have risen to dominance relatively quickly over the last 100 million years. Recently completed whole diatom genomes have revealed a wealth of information about the evolutionary origins and metabolic adaptations that have led to their ecological success. A major finding is that they have incorporated genes both from their endosymbiotic ancestors and from marine bacteria by horizontal gene transfer. This cacophony of genes encodes novel capacities for metabolic management, for example, allowing the integration of a urea cycle into a photosynthetic cell (Bowler et al., 2013).

Transcriptomics has become a popular way to approach the problem of analyzing phytoplankton gene expression (Ashworth et al., 2013; John et al., 2009; Zielinski, 2014). For eukaryotic phytoplankton, size fractionation of phytoplankton communities or selection of polyA-tailed transcripts (or both) have been used to isolate the eukaryotic fraction of the metatranscriptome. However, this will not enable the capture of mitochondrial or plastid transcripts. Indeed, sometimes plastid transcripts have polyA tails added to identify them for specific degradation (Ward, 2008). After capture on polydT, the RNA is amplified (usually by MessageAmp II, Applied Biosystems). The amplified RNA is then converted to cDNA followed by a second strand synthesis, library construction, and Illumina sequencing.

Proteomics (the study of the sum total of proteins in a cell, also termed the proteome) methods are becoming more widespread in the study of phytoplankton gene expression (Rahman, 2014). The rationale here is to measure production of proteins during gene expression events and not transcripts, which may not be translated into a functional protein. However, the presence of a protein in a host does not mean that protein is catalytically active. This requires a test of its functional capabilities which may not be easily measured.

3. Case Histories

3.1. Carbon

The study of important gene functions in any organism depends on the completeness of the database for that particular gene and the taxa that might contain it. In 1992, the *rbcL* gene seemed particularly appropriate because of the relative conservation of sequence of this gene, and the significance of c-fixation in marine euphotic communities (Pichard, 1993). This paper used the *rbcL* gene of *Synechococcus* PCC 6301 as a probe of DNA and RNA blots to calculate mRNA/DNA ratios, or what was termed “Gene expression per gene dose”. At that time it was thought *Synechococcus* PCC 6301 *rbcL* was representative of all phytoplankton but in reality only reflected the Form IB group of RUBISCOs (ribulose bisphosphate carboxylase/oxygenase) (green algae and higher plants). Therefor this early attempt to study *rbcL* expression in all phytoplankton would have missed diatoms, haptophyes, and prymnesiophnyes (FormID), *Prochlorococcus* and PE-containing *Synechococcus* (FormIA; (Paul et al., 2000) and dinoflagellates (either FormID or FormII (Tabita, 1999)). Using deck-top incubators, this approach yielded a clear pattern of diel variation in transcription of *rbcL* with the maxima at dawn and early daylight hours and minimal in the dark (Pichard, 1993). Further work using a *Prochlorococcus* strain in culture or natural populations in the Gulf of Mexico found that the diel pattern of *rbcL* transcription was also rhythmically entrained (Pichard et al., 1996). That is, changing natural populations or *Prochlorococcus* in culture from light:dark incubations to continuous illumination resulted in the same entrained pattern of transcription.

The physiological and transcriptomic changes during typical diatom growth in batch culture was studied in *Thalassiosira pseudonana* (Ashworth et al., 2013). Four principal states were investigated: (i) "dawn," following 12 h of darkness; (ii) " dusk," following 12 h of light; (iii) exponential growth and nutrient repletion; and (iv) stationary phase and nutrient depletion. Literally thousands of genes showed increased transcription during the dawn period, including RUBISCO. Reproducible changes in transcription of sensory, signaling, and regulatory functions accompanied the four cell-state transitions, yielding a preliminary road map of how the diatom gene regulatory network operates (Ashworth et al., 2013).

CO_2 fixation in river plumes is a fascinating story of nutrients, light availability, frontal boundaries, and large gradients in salinity. They often dictate phytoplankton C metabolism and modulate phytoplankton production many miles from shore. Our studies have taken us to the Mississippi River Plume (MRP), Orinoco River Plume (ORP), and the Amazon (ARP). The MRP often just loops westward from the Crowsfoot of the Mississippi River delta and results in a zone of hypoxia on the Texas and Louisiana shelf (Rabalais et al., 2002). However when entrained in the Loop Current in the GOM, the plume forms a 10 m deep feature that is highly visible by water color (Figure 2C). Analysis of *rbcL* cDNA libraries indicated that the plume was divided into several phytoplankton domains. Diatoms dominated the most inshore, highly productive waters of the plume. Traveling offshore down the axis of the plume there is a community of PE-containing *Synechococcus,* followed by a second diatom community. The bluewater non-plume stations were dominated by high light *Prochlorococcus* cells (Wawrik and Paul, 2004).

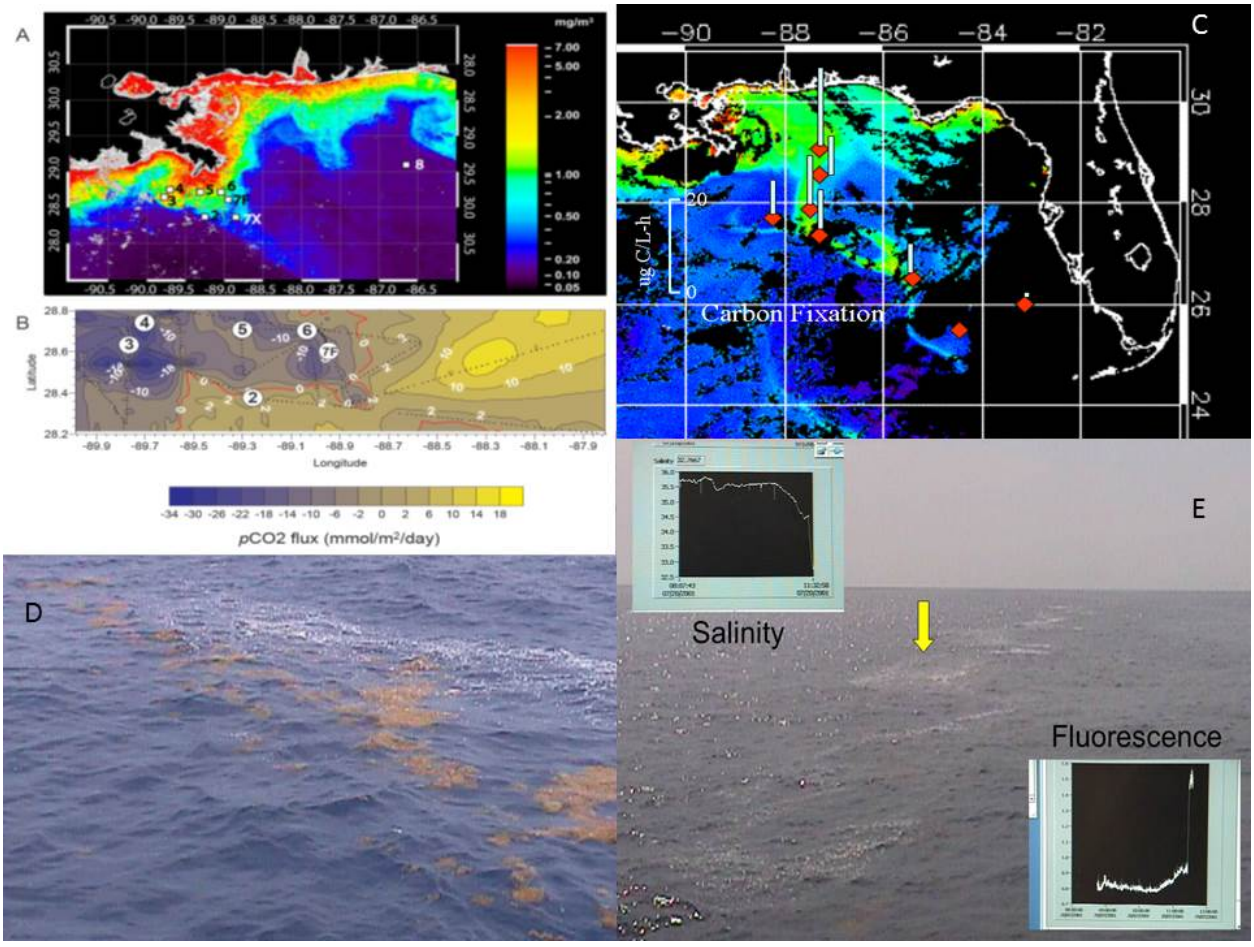


Figure 2. Panel A: MODIS satellite chlorophyll a image of MRP showing station locations. Panel B: Plot of CO_2 Flux as a function of station location. Panel C:SeaWiFS chla satellite image of the NE GOM plus carbon fixation rates (Vertical bars) for stations inside and outside the MRP. Panel D and E: The frontal boundary of the MRP in the nearshore (E) and the bluewater plume(D). Note collection of Sargassum at the frontal boundary at the bluewater plume.

A comparison of the MRP to the Orinoco River Plume (John et al., 2012)indicated that these environments were fundamentally different. The MRP is fueled by nutrients drained from the agriculturally rich drainage basin whereas the Orinoco drains natural mangrove rich lands that results in an order of magnitude less chlorophyll, photosynthesis, nutrient uptake, and total rbcL mRNA levels . As shown in other environments, there was a strong diel cycle in rbcL mRNA levels and PS carbon fixation. In both environments there was a lag between rbcL mRNA and carbon fixation, but the lag was longer in the MRP than the ORP. This lag was primarily in the >2 [illegible] fraction of the MRP phytoplankton. These results were explained by larger eukaryotic phytoplankton having more mechanisms available to modulate carbon fixation gene expression than the smaller, principally prokaryotic photoautotrophs, being transcriptionally regulated (John et al., 2012).

Equally important in PS carbon fixation as *rbcL* which in part with *rbcS* catalyzes the first step in the Calvin-Bensom-Bassham cycle is the molecular machinery to supply RUBISCO with CO_2, termed the carbon concentrating mechanisms (CCMs). In most prokaryotes that contain RUBISCO, the CCM genes are cistronic with *rbcL* and *rbcS* (John et al., 2006). The capacity of phytoplankton to express CCM genes is regulated by environmental factors (Beardall and Giordano, 2002). Some factors such as photon flux can instantaneously change CCM-function without the need to make transcriptional changes. Those environmental parameters that cause changes in the availability of dissolved CO_2 in the media surrounding the cells act at the transcriptional level (Beardall and Giordano, 2002).

Another player in the autotrophic carbon acquisition game is the bicarbonate transporters and the carbonic anhydrases. In the bloom forming cyanoabcterium *Microcystis aeruginosa* there are the ATP-dependent bicarbonate uptake system BCT1 and several carbonic anhydrases (CAs). Strains isolated had the high-flux bicarbonate transporter BicA or the high-affinity bicarbonate transporter SbtA. Growth experiments showed that strains with sbtA performed better at low inorganic carbon (Ci) conditions, whereas strains with bicA performed better at high Ci conditions (Sandrini et al., 2014). Both these genes were on the same operon, and were believed to be regulated by a LysR-type transcriptional regulator.

Carbon concentrating mechanisms and CO_2 fixation pathways are thought to play critical roles in adaptation to increasing pCO_2 resulting from global climate change. *Emiliani huxlyi* is a coccolithiphore of global importance, fixing CO_2 as well as making $CaCO_3$ plates. Acclimation experiments suggest negative effects of warming and acidification on coccolithophore calcification, but the ability of these organisms to adapt to future environmental conditions is not well understood (Benner et al., 2013). Cells exposed to increased inorganic carbon content and calcification rate under warm and acidified conditions compared with ambient conditions, indicating organic carbon content and primary production did not show any change. In contrast to findings from short-term experiments, the results suggest that long-term acclimation or adaptation could change, or even reverse, negative calcification responses in *E. huxleyi* and its feedback to the global carbon cycle. Transcriptome analysis revealed that genes thought to be essential for calcification are not those that are most strongly differentially expressed under long-term exposure to future ocean conditions. Rather, differentially expressed genes observed here represent new targets to study responses to ocean acidification and warming (Benner et al., 2013).

3.2. Nitrogen

The interactions between Carbon and nitrogen in phytoplankton are complex. Early research efforts, however, often did look at nitrogen independently of carbon or other macronutrients.

Smith et al (1992) used now classical methods to detect diatom response to nutrient flux and increase in PAR one might find in a coastal upwelling. After growing cultures of *S. costatum* under N-limitation and lowlight, shifting into replete nitrate and non-limiting illumination the cells resulted in an increase principally in nitrate uptake and nitrate reductase activity (NRA). Diel fluctuations in NRA were not observed in patterns of change in nitrate reductase protein (Smith et al., 1992).

Regulation of nitrate reductase (NR) was monitored in cultures of *Thalassiosira weissflogii* by monitoring NR protein immunologically as well as nitrate reductase activity. Cells grown on ammonium produced neither NR protein nor activity. Because the RNA synthesis inhibitor Actinomycin D and the protein synthesis inhibitor cycloheximide inhibited both NR activity and protein, this argued strongly for regulation of NR at the transcriptional level (Vergara et al., 1998).

The first nitrate transporter (NAT) to be cloned was derived from the diatom *Cylindrotheca fusiformis* Reimann et Lewin. Actually three homologs were discovered, only varying in 18 of 1446 nts. NAT mRNA levels were high in the presence of exogenous nitrate but highly repressed when cells were grown in the presence pf NH_4CL or NH_4NO_3. NAT transcript abundance did not occur continuously through the cell cycle, but rather had elevated levels at the beginning of G1 and again at the S-G2 interface. The abundance of silica also influenced the transcription of the NAT genes (Hildebrand and Dahlin, 2000). In *Skeletonema costatum*, phosphate limited cultures had lower levels of the high-affinity nitrate transporter (Nrt2) transcripts than those growing with adequate P (Liu et al., 2013). Compared to the low affinity transporter, the high-affinity Nrt2 transcript levels were *inversely related* to nitrate concentrations below 40 µmol/L (=40 nM; (Liu et al., 2013))

Glutamine synthetase is a key enzyme in nitrogen assimilation as well as a key regulator of cellular N and P metabolism. Transcript abundance of glnII (the gene that encodes the GSII isoenzyme), as well as GSII enzyme activity increased in *Skeletonema costatum* (Greville) Cleve cells growing on nitrate but not

taking up ammonium (NH4+; (Takabayashi et al., 2005). Thus GSII transcript abundance could provide a measure of new production in the oceans.

The impact of N-starvation on the metabolic and lipidomic profiling of 97 low-molecular weight compounds from the primary metabolism and 124 lipid compounds in *Thalassiosira pseudonana* was determined (Bromke et al., 2013). Storage lipids (triglycerides) accumulated as well as TCA cycle intermediates (particularly citric acid which increased 10-fold) in N-starved cultures, whereas amino acid pools were depleted. The increase in TCA intermediates paralleled increases in expression of the TCA *cycle genes.* Proline, believed to be the major osmoprotectant under N replete conditions was replaced by dimethylsulfoniopropionate under conditions of N-starvation. Therefore diatoms can alter their metabolic fluxes and metabolite abundances to adapt quickly and efficiently to environmental change (Bromke et al., 2013).

3.3. Phosphorus

Not only is phosphorus an essential macro-nutrient for phytoplankton growth, it can play an important role in harmful algal bloom initiation. Alkaline phosphatase (AP) plays a vital role in organic P utilization. Lin et al.(2012) isolated the AP gene from the HAB *Karenia brevis* and found it to be a cell surface associated protein most closely related to the AP of another toxic dinoflagellate, *Amphidinium carterae.* When *K. brevis* cells were cultivated in low DIP, AP enzyme activity increased 5-6 fold (Lin et al., 2013).

P-starvation did not result in a transcriptional response in *K. brevis* relative to cells grown under P-replete conditions.(Morey J.S.; Monroe, 2011) . However, shift from depleted to replete P conditions resulted in significant changes in 4% of the genes on the *K. brevis* microarray. The earliest responding genes were for pentatricopeptide repeat (PPR) proteins, which increased in expression up to 3-fold by 1 h following nutrient addition. PPR proteins are nuclear encoded proteins involved in chloroplast and mitochondria

RNA processing. The nuclear encoded gene transcripts in *K. brevis* all contain a 5' trans-spliced leader (SL) sequence suggestive of regulation by posttranscriptional mechanisms.

A proteomics study of the effect of phosphate deficiency on the marine HAB-forming alga *Aureococcus anophagefferens*. Under times of P-scavaging, relative increases in the protein levels of a phosphate transporter, 5'-nucleotidase, and alkaline phosphatase. P-starvation also resulted in the replacement of phospholipids with sulfolipids (Wurch, 2011).

3.4. Iron

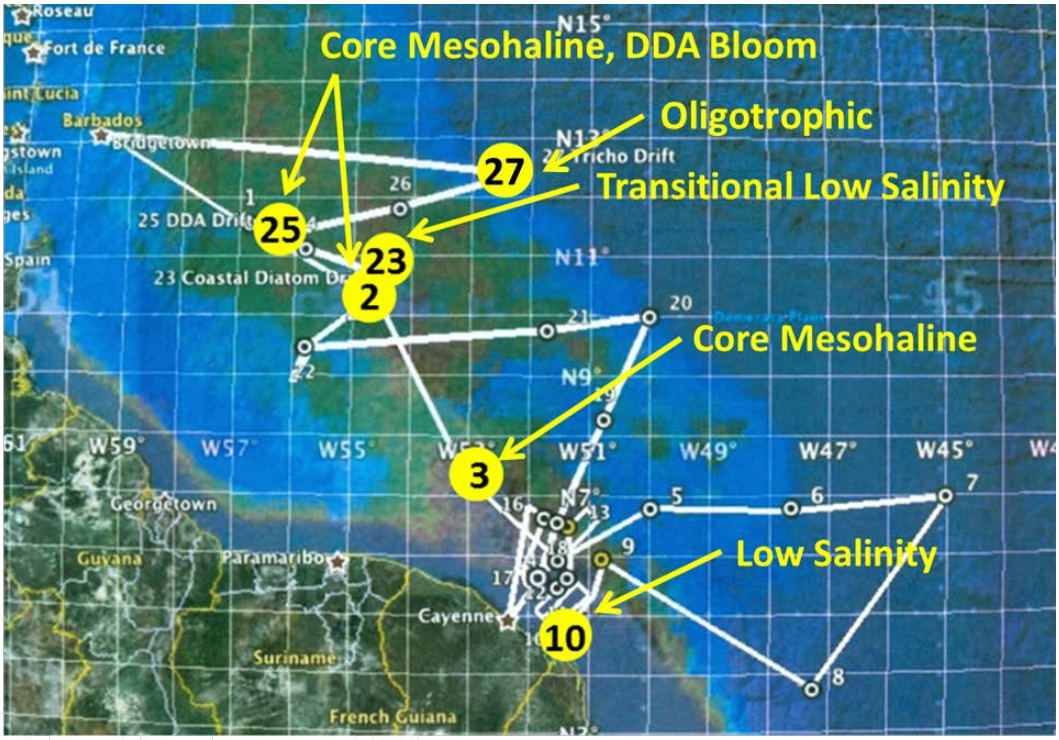
Iron has been shown to limit phytoplankton growth, production, or perhaps more correctly gene expression in many portions of the world's oceans. *Prymnesium parvum* was grown under Fe-deplete (0.0025 nM) and Fe-rich (0.05 nM) conditions, and proteomic responses of the organism to Fe conditions were compared (Rahman, 2014). Seven proteins were highly expressed under Fe depleted conditions while one protein was over expressed in Fe-replete conditions as determined by SDS poly acrylamide gel electrophoresis. The proteins were identified by matrix-assisted laser desorption-ionization-time of flight-mass spectrometer (MALDI-TOF-MS) analysis. Under Fe-deplete conditions, *P. parvum* increased the biosynthesis of ATP binding cassette (ABC) transporters, flagellar associated protein (FAP), and phosphoribosyl-aminoimidazole-succinocarboxamide synthase, proteins believed to play a role in Fe-acquisition in phytoplankton. Also apparently upregulated during Fe-depletion were ribulose bisphosphate carboxylase (RUBISCO), malate dehydrogenase, and two Fe-independent oxidative stress response proteins, manganese superoxide dismutase (MnSOD) and Serine threonine kinase (STK) (Rahman, 2014). The investigators conclude that *P. parvum* has several gene expression options in responding to Fe depletion as compared to a single gene or operon response.

More than any other group of phytoplankton, large diatoms are particularly susceptible to iron limitation. Iron enrichment experiments typically result in large diatom blooms. A microcosm iron enrichment experiment from the mixed layer of the northeastern Pacific Ocean was performed and transcriptomes

generated for enriched and unenriched treatments (Marchetti et al., 2012). In the iron-amended microcosm; transcripts of diatom genes required for synthesis of photosynthesis and chlorophyll components, nitrate assimilation and the urea cycle, and synthesis of carbohydrate storage compounds were significantly overrepresented. Oddly enough, samples receiving supplemental iron had an under-representation of eukaryotic diatom rhodopsins , implying that rodopsins might play a role in photic energy transduction under low iron levels. That is the iron depleted oceanic phytoplankton might curtail their iron-dependent photophosphorylation (cytochromes, etc.) and derive energy from rhodopsin-like activities (Marchetti et al., 2012).

A comparison of a coastal diatom *Thalassiosira pseudonana*, to its oceanic relative *Thalassiosira oceanica* indicated that the latter was highly tolerant to iron limitation compared to its coastal sibling. Genomic analysis of the two diatoms indicated that the gene encoding ferrodoxin (petF) was in the nucleus of *T. pseudonanna* but had been transferred to the nucleus in *T. oceanica* (Lommer et al., 2010). Ferrodoxin can be replaced by flavodoxin during iron limited growth. Presumably the nuclear petF location of *T. oceanica* enables this organism to have a competitive advantage in Fe economy during iron limited growth.

3.5. Patterns of Gene Expression



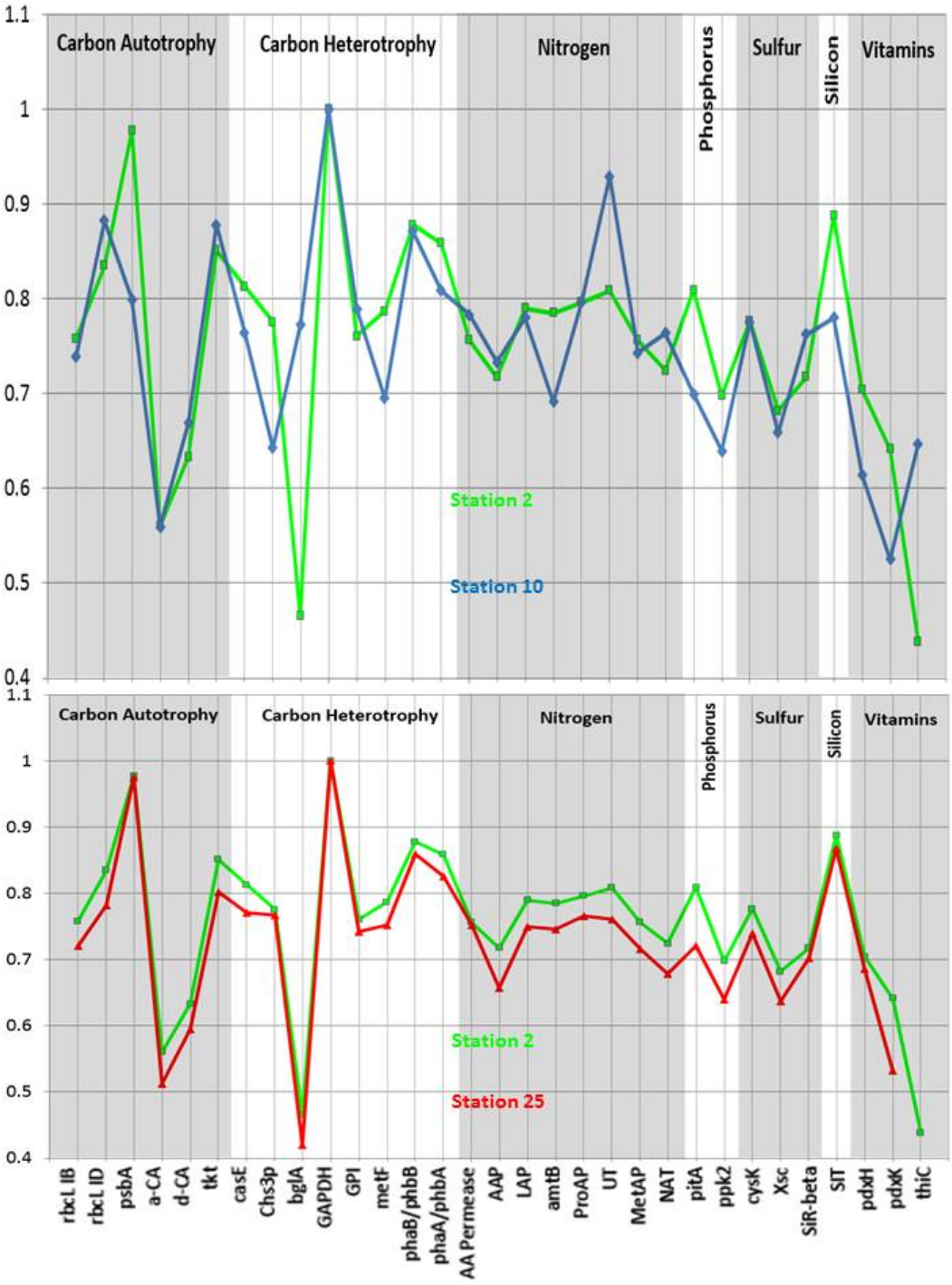
There is value in studying patterns of gene expression by gene cohorts in response to differing

environments, environmental perturbations, and time. For example, our interrogation of eukaryotic planktonic meta-transcriptomes through spatial and temporal salinity gradients of the Amazon River Plume (ARP) indicated that stations where surface samples were defined by similar nutrient, sunlight, and salinity regimes resulted in similar patterns of gene transcription (Zielinski, 2014)

Figure 3. Cruise track of the R/V Knorr in the ARP 2010. Meta-Transcriptomic samplings occurred at stations indicated by yellow circles.

Station 10 was a low salinity, nitrate-fueled diatom bloom. Station 10 was a low salinity, nitrate-fueled diatom bloom. Stations 2 and 25 were both diatom diazotroph associations stations (DDAs), with unmeasurable nitrate and an intermediate salinity. These two stations were sampled nearly a month apart, yet retained a similar biogeochemical and metatranscriptomic identity (Zielinski, 2014).

Figure 4. Analysis of patterns of gene expression (>2 um particle size) of polyA tailed transcripts from stations 2 and 10 (top panel) and 2 and 25 (station locations in Fig. 3). Thirty-one genes (X-axis) were BLASTED against each station's transcriptomes. Then each transcriptome was normalized to the transcript abundance for glyceraldehyde phosphate dehydrogenase (GAPDH), a common housekeeping gene in both autotrophs and heterotrophs (which yields the Y-axis values, ranging from 0 to 1). Notice the nearly identical patterns of expression between Sta 2 and 25 and the disparate patterns between 2 and 10).



Analysis of 130,00 ESTs from *Phaeodactylum tricornutum* cells grown in 16 different conditions have resulted in similar expression profiles and identified transcripts differentially expressed in response to specific treatments (Maheswari et al., 2010). Functional annotation of these transcripts provides insights into expression patterns of genes involved in various metabolic and regulatory pathways and into the roles of novel genes with unknown functions. Specific growth conditions resulted in greater gene diversity, known gene product functions, and over-representation of rare and/or unknown transcripts. When comparative expression analysis was done with *Thalassiosira pseudonana*, it was possible to identify unique diatom genes that were specifically regulated to a particular set of conditions. This common transcriptomic response enabled studies of gene function, genome annotation, and the genomic basis of species diversity and phenomics (Maheswari et al., 2010).

4. Omics and the Future of Phytoplankton Gene Expression

Years ago the study of the regulation of production of an enzyme or several enzymes in any biological system was called "Enzymology"(the term "proteomics" had yet to be invented). When such processes were studied in the field investigators were armed with one or two assays and correlated gene-function (a term for expression) to one or several processes or environmental variables. Things were simple. Today proteomics papers are becoming commonplace that look for the over-representation of transcripts (what was previously termed upregulation) in response to an environmental perturbation (Fe limitation, increase in pCO_2, etc.) and hundreds of transcripts arise as potentially important candidates in the regulation of a process. The conclusions? The organism has multiple genes, pathways, and processes responding to the stimuli (Dyhrman et al., 2012). Are all processes needed to elicit the gene function of concern or are the secondary processes fortuitously co-regulated?

The core problem in making sense of gene expression in phytoplankton is the lack of functional annotation research to truly identify the tremendous number of highly expressed or repressed genes that are either responding to a perturbation or resulting in a different phenome (or both). Although

bioinformatics has become extremely sophisticated through PFAM, hidden Markov models, and like analysis in the attempt to correctly annotate gene function, there is still a tremendous need to do what was previously called “genetics”. That is, through gene knockout/knockdown analysis to pinpoint gene function for an orfan, a conserved hypothetical ORF, and the like. However, genetic systems or means of site directed mutagenesis and manipulation are lacking in most environmentally significant or relevant taxa. Using the diatom *Phaeodactylum tricornutum* it has been demonstrated that gene silencing mechanisms exist to trigger knockdowns and knockouts to identify gene function (De Riso et al., 2009). Using either inverted repeats or antisense constructs of selected target genes it has been possible to silence a GUS reporter gene as well as the knockdown of endogenous phytochrome (DPH1) and cryptochrome (CPF1) genes. Initial molecular analyses reveal that targeted downregulation likely occurs through transcriptional and post-transcriptional gene silencing mechanisms (De Riso et al., 2009). This approach, albeit tedious, blazes the trail for reverse genetics analysis to understand the biology and ecology of diatoms.

Omics data can be organized along the flow of biological information: genomics, transcriptomics, proteomics, metabolomics, and phenomics. Briefly, genomics is the total genome sequence of an organism, including ORFs, regulatory elements, and non-coding RNAs. Transcriptomics is the sum total of all the transcripts in the organism. Proteomics is the sum total of all proteins in the cell, and metabolomics refers to the intracellular pools of small mw metabolites. The sum total of all observable traits in an organism is the field of Phenomics, also termed the sum of all other omics (Tian, 2011).

For a single organism, this approach makes sense, even though the integration of all omics in one database has yet to occur for any organism. As we see the various Meta-omics (ie Meta-genomics, meta-transcriptomics , meta-proteomics), one must consider yet another layer of organization, meta-Phenomics. Meta-phenomics is the sumtotal of all observable phenotypes in a system. The interaction of phenotypes in an environment is a definition of ecology. On a global scale, could the sum total of the various ecological systems on this planet be called the Eco-nome? If so, the term for the field would be

"Economics", ironically sharing the name for "**A social science that studies how individuals, governments, firms and nations make choices on allocating scarce resources to satisfy their unlimited wants."**

More correctly, the aggregate of all ecological interactions on the planet would be the "Ecolome".

5. Web Resources

Transcriptomics

The Moore Foundation initiated a Marine Microbial Eukaryote Transcriptome Sequencing Project (http://marinemicroeukaryotes.org). As transcriptomes emerge from this initiative, the entire marine eukaryotic phytoplankton database will exponentially grow.

Proteomics

ExPASy bids itself as a Bioinformatics Resource Portal and contains links to a myriad of databases amd web tools. http://www.expasy.org/proteomics

Metabolomics

Perhaps the largest collection of plant and microbial metabolomics web resources: http://www.bmrb.wisc.edu/metabolomics/external_metab_links.html

Phenomics

An interesting initiative of Avotol is Next Generation Phenomics for the Tree of Life. A multi-institutional collaboration to increase the use of phenomic data for phylogenetic tree-building by using: Crowd Sourcing, Natural Language Processing, and Computer Vision. http://avatol.org/ngp/

6. Acknowledgements

This research was supported by the Gordon and Betty Moore Foundation as part of the River Ocean Continuum of the Amazon (ROCA) project (Grants GBMF #2293 and #2928). Special thanks to Brian Zielinski and Patricia Yager for helpful discussions.

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Chapter 4

Emiliania Huxleyi

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Emiliania huxleyi in the genomic era

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1. Abstract

Emiliania huxleyi is a globally abundant calcified marine phytoplankton species that plays a significant biogeochemical role in modern oceans. The species has become an important model organism, with extensive research conducted on its responses to carbon chemistry, light regimes and nutrient dynamics. In the years since the seminal review on *E. huxleyi* by Paasche (2002), the field of microbial oceanography has been revolutionized by the increasing application of genomic and post-genomic technology. Paasche intended his review

as a starting point for future molecular studies on coccolithophores, a field in 2002 that was described as "barely in its infancy." We revisit this review and use it as a framework to describe how our knowledge of this species has progressed since these tools have become more commonly applied. We focus on the same key areas as Paasche, namely how the morphology, diversity, life cycle and physiology of this species affects calcification and to a lesser extent, calcification-photosynthesis interactions. We use these to present a framework for future research and suggest directions to help answer many of the questions still posed regarding the biology of this globally important yet seemingly enigmatic organism.

2. Introduction

Coccolithophores are single celled, eukaryotic marine phytoplankton distinguished by their ability to produce calcite plates (coccoliths) that cover the exterior of the cell. In the modern ocean, coccolithophores are one of the main sources of calcite ($CaCO_3$) to deep-sea sediments (Milliman 1993; Westbroek et al., 1993) and may fix up to 20% of carbon in some systems (Poulton et al., 2007). *Emiliania huxleyi* (Lohmann) Hay et Mohler, a species that first emerged between 265,000 to 290,000 years ago (Thierstein et al., 1977; Raffi et al., 2006), is the most abundant coccolithophorid in modern oceans and often dominates counts from oceanic sediment traps (Beaufort et al., 2007). *E. huxleyi* has a wide distribution, forming between 30-70% of mid-latitude and 10-20% of low-latitude coccolithophore floras (Westbroek et al., 1989). The species also forms extensive subpolar blooms (Brown and Yoder, 1994; Iglesias-Rodriguez et al., 2002; Figure 1). It is this ecological and biogeochemical importance, as well as the ease with which *E. huxleyi* can be cultured, that have made the species a suitable model organism for studies on coccolithophore biology (Westbroek et al., 1993). Consequently, *E. huxleyi* ecophysiology has been studied intensively and was the focus of an extensive review on the species by Paasche in 2002. Since then, a wealth of knowledge has emerged warranting a re-evaluation of the biology and physiology of this important organism. With this in mind, we intend this review to be a

companion text to Paasche (2002). However, befitting a review on such a biogeochemically important species, not all aspects can be covered in a book chapter, so we only focus on those in which significant progress. Similar to Paasche, fields such as *E. huxleyi*-virus interactions, alkenones and DMS production are not discussed here (the latter subject is touched upon in chapter 7).

3. Genetic and morphological diversity

Evidence was already beginning to come to light that *E. huxleyi* was a morphologically and genetically diverse species in 2002, when Paasche compiled his review. This issue of variability likely affects most factors regarding the responses of this species to differing environmental dynamics and extensive efforts have been directed to understanding intraspecific diversity in recent years. A plethora of genomic studies and the recent publication of the *E. huxleyi* genome (Read et al., 2013) have helped us appreciate that this species may be an extremely complex driver of oceanic biogeochemistry.

3.1. Morphotype diversity

At present, seven different morphotypes of *E. huxleyi* are typically recognized; A, B, B/C, C, O, R and corona (Figure 2), based on immunological, biochemical, geographic, physiological, genetic and morphometric differences (e.g. van Bleijswick et al., 1991; Young and Westbroek, 1991; Young et al., 2003; Hagino et al., 2005; Hagino et al., 2011). Some authors have raised these morphotypes to distinct varieties (var. *huxleyi* [type A], var. *pujosae* [type B], var. *kleijniae* [type C], Medlin et al., 1996; var. *aurorae* [type B/C], Cook et al., 2011). Type D is an additional morphotype occasionally mentioned in the literature (Findlay and Girardeau et al., 2000; Mohan et al., 2005) but it is likely that this exists as an artifact caused by *in situ* seawater chemistry, collection techniques, microscopy or fixation.

Recent evidence supports splitting morphotypes of *E. huxleyi* into two groups: A (composed of types A, R and corona) and B (composed of types B, B/C, C and O) based on shape and

degree of calcification (Young, pers. comm.). Group B cells are all lightly calcifying and include the recently defined type O that possesses coccoliths similar to type B but with an open central area (Hagino et al., 2011). Type O is typically easier to classify in culture but in the field it is more difficult to discern from other morphotypes since it often co-occurs with type B, whilst types B/C and C often have open central areas. This means some early field distribution reports may require reappraisal. There is some variation in calcification within group A, which includes R, a distinct morphotype that resembles cells of the genus *Reticulofenestra* (Young et al., 2003) and corona, similar to A but with a protruding surface. Morphotype A has also been observed as "over calcified" in some waters, bearing coccoliths with a closed central area (Smith et al., 2012).

It is becoming clear that different morphotypes of *E. huxleyi* may have differential distribution patterns. B appears to be a Northern hemisphere type that is particularly common in the North Sea and around the British Isles (van Bleijswick et al., 1991) but it is not known if this morphotype is more geographically extensive (Hagino et al., 2011). Type A is known to have a global distribution, appearing in samples from both hemispheres (e.g. Cubillos et al., 2007; Beaufort et al., 2011; Smith et al., 2011; Poulton et al., 2011). Meanwhile, B/C (*sensu* Young et al., 2003) has so far only observed in sub-polar (Hagino et al., 2005) and Southern hemisphere waters (Cubillos et al., 2007; Finlay and Girardeau, 2000; Mohan et al., 2005; Henderiks et al., 2012; Hinz et al., 2012) with an apparent preference for nutrient rich but colder (<10°C) waters (Poulton et al., 2011). Moreover, Southern Ocean B/C strains are genetically distinct from Southern Ocean type A isolates and possess a different pigment profile (Cook et al., 2011), suggesting differences in morphotype ecophysiology. Distinct hydrographic preferences (Beaufort et al., 2011), additional genetic differences between type A and B (Schroeder et al., 2005), and the presence of multiple unique genotypes of type A (Schroeder et al., 2005), B/C and R (Cook et al., 2011) indicate that *E. huxleyi* is a pseudo-cryptic species complex harboring extensive genetic variability.

3.2. Genetic diversity

Since the 2002 review, the application of genomic tools and genome sequencing has become seemingly commonplace. High levels of intraspecific diversity originally proposed by Brand (1982), and suggested by RAPD fingerprinting (Medlin et al., 1996), have now been confirmed using a variety of methods. For example, Iglesias-Rodriguez and co-authors (2002) conducted AFLP on nine strains to suggest that there are distinct Northern and Southern hemisphere populations. Hagino et al. (2011) investigated the genetic variability of 39 strains, isolated from a diversity of ocean basins, using mitochondrial *cox1b* and *atp4* genes to find two clades differentiated by water temperature and phosphate concentration. Denaturing gradient gel electrophoresis using the calcium binding *GPA* genes (see section 3.3) suggested that *E. huxleyi* blooms in the North Sea were dynamic and diverse assemblages (Martinez et al., 2012). Meanwhile, microsatellite analysis on 85 global isolates (Iglesias-Rodriguez et al., 2006) and 273 Southern hemisphere strains (Cook et al., 2013) indicated that *E. huxleyi* was a species complex composed of numerous genetically diverse phenotypes selected by environmental pressures.

The recent publication of the *E. huxleyi* reference genome of CCMP1516 accompanied with the sequences of 13 additional strains confirmed the presence of vast levels of diversity at the molecular level. This suggested that the species actually exists as a pan-genomic complex, with some strains only exhibiting 54-77% similarity to the reference genome (Read et al., 2013). Moreover, the presence and absence of genes between strains was exhibited, in addition to variable copy numbers of genes involved in nutrient acquisition and metabolism. This lead Read and co-authors to make the same conclusion as Paasche; namely that a single strain will never be a perfect representative for all strains. More so than ever, *E. huxleyi* should be considered a plastic, rapidly adapting and resilient entity. Indeed, Kegel and co-authors (2013) recently discovered that gene content sometimes varies by up to 30% in different strains of *E. huxleyi*, leading them to conclude that the species is undergoing a rapid evolutionary radiation and should be described as a species-morphotype complex. It is likely that this strain variability underlies many aspects of the various reported responses of

coccolithophores to ocean acidification (Langer et al., 2009; Ridgwell et al, 2009) and other environmental variables (section 5). Diversity observed in other coccolithophore species (Geisen et al., 2004; Quinn et al., 2004) suggests that this may be a common characteristic for marine eukaryotic phytoplankton and is a feature that has been recognized in marine bacteria (e.g. SAR11, Grote et al., 2012).

Ultimately, it remains to be seen whether variations in morphotype and the underlying genetic diversity of *E. huxleyi* have biogeochemical ramifications. Although there is considerable overlap in the size of coccoliths produced between morphotypes (Poulton et al., 2011), evidence is beginning to come to light that different morphotypes can have distinct impacts on regional calcification rates. For example, blooms of B/C morphotype cells have significantly lower $CaCO_3$ production rates compared to blooms composed of other morphotypes (Poulton et al., 2013), theoretically affecting surface-deep sea CO_2 and $CaCO_3$ fluxes. Additionally, the levels of genetic diversity exhibited by the *E. huxleyi* species group suggest that it is an exceptionally complex driver of elemental cycling. For example, comparisons of the genomes of different strains unveiled variable distribution of nitrate reductase (Read et al., 2013), and substantial inter-strain functional diversity was exhibited with respect to differences in enzymatic activity (Reid et al., 2011). Therefore the questions now needing to be taken into consideration are 1) what is the underlying cause of this level of variability and, 2) what are the biogeochemical implications?

4. Life cycle considerations

Although Paasche (2002) already reviewed the existence of different types of *E. huxleyi* cells associated with life cycle stages and the potential biogeochemical differences between them, this has been a factor often neglected in both field and laboratory studies. However, recently life cycles in marine phytoplankton are beginning to be reconsidered (see von Dassow and Montresor, 2011). Three cell types are still recognized for *E. huxleyi*; C (coccolith forming), N (naked) and S (scaly) but to our knowledge, there have been no further mention of

amoeboid cells in the literature since the review of Paasche. As before, it is generally accepted that C cells are diploid adult stages, N cells are mutant diploid non-calcifiers and S cells are haploid, non-calcifying, motile gametes. Anecdotally, Houdan et al. (2004) proposed that meiosis might relate to circadian rhythms or seasonality, whilst Frada et al. (2008) suggested that reactive oxygen species were responsible, however mechanisms for phase shifts remain unknown. Microsatellite analyses also suggested that the species may regularly undergo sexual reproduction (Iglesias-Rodriguez et al., 2006; Cook et al., 2013). It is now generally accepted that coccolithophores alternate asexuality by binary fission with sexual reproduction by syngamy (Klaveness, 1972; Green et al., 1996), although the latter process has not yet been observed. Each stage is able to reproduce vegetatively in an indefinite manner, as confirmed by culture studies (Billard and Inouye, 2004), and haploid cells may persist at low (<0.l cells mL^{-1}) levels throughout the year (Holligan et al., 1993).

So far, the distribution of S (haploid) cells in the environment is unknown, since they are difficult to differentiate from small flagellates and are not well preserved using classical techniques (von Dassow and Montresor, 2011). Following early attempts by Campbell et al. (1994) to detect haploid cells in the field using immunofluorescence, Frada et al. (2006) developed a method, combining polarized optical microscopy with fluorescent in situ hybridization, to enable the detection of both haploid and diploid coccolithophore cells. This was used to confirm that haploid cells of *E. huxleyi* were present within Norwegian fjords (Frada et al., 2012) but little else is known about the distribution of these cells in the environment.

It is likely that haploid and diploid cells of *E. huxleyi* represent phases that are evolutionary shaped to occupy specific niches in different environments. Unlike calcified diploid cells, uncalcified haploid cells are motile, have a stronger defense response to grazers (Kolb and Strom, 2013), are resistant to viral attack (Frada et al., 2008), and may also be more sensitive to light (Houdan et al., 2005) and high CO_2 (Fiorini et al., 2011). At the molecular level, succinct differences have also been observed between haploid and diploid cells of *E. huxleyi*.

For example haploid cells exhibited higher transcriptional levels of genes associated with nitrogen and carbon metabolism, as well as vitamin synthesis (Rokitta et al., 2011). Expressed sequence tag analysis also indicated dramatic differentiation (only ~50% of transcript clusters shared) between the two cell phases, with haploid cells producing more transcripts associated with light sensing and signal transduction (von Dassow et al., 2009).

Despite differentiation at the transcriptional level, there may ultimately be no difference in functionality between these phases, since Reid et al. (2011) saw no difference between haploid and diploid cells of the same strain of *E. huxleyi*. However, that is not to say that further investigations will not reveal metabolic differences between these strains when more loci are investigated. Additionally, there is some evidence that ploidy level may affect biogeochemistry. For example, haploid cells produce fewer transparent exopolymer particles (TEP) than diploid cells (Pedrotti et al., 2012; Van Oostende et al., 2013), but exude a higher concentration of dissolved carbohydrates (Van Oostende et al., 2013). This may relate to calcification status, since coccoliths have an organic coating that may be transformed to form TEP (Godoi et al., 2009). A lower concentration of TEP in the haploid stage could alter export efficiency by preventing dissolved polysaccharides being coagulated and exported to depth (see Engel et al., 2004). While it is clear that cell stage could therefore have substantial biogeochemical implications, this requires further investigation. To better understand ocean carbon cycling, we must now turn out attention to consider when, where and why different cell phases arise.

Models have suggested that haploid-diploid lifecycles persist when niche differentiation and haploid resistance to competition is sufficient to compensate for the benefits of being diploid (namely higher survival and reproduction; Hughs and Otto, 1999). This suggests that *E. huxleyi* populations exist through an evolutionary stable strategy (*sensu* Maynard Smith and Price, 1973) and significant differences between haploid and diploid phases enables their persistence. Accordingly, diploid cells may be able to occupy numerous niches, since they have a richer transcriptome (von Dassow et al., 2009) but the additional benefits of motility,

such as locomotion and ability to generate thin nutrient filaments *via* turbulence (Taylor and Stocker, 2012), notwithstanding the different carbon and nitrogen metabolic requirement of haploids (Rokitta et al., 2011), enables them to exist in environments that are not as advantageous to the diploid phase. Additionally, the smaller size of haploid cells results in a greater surface area to volume ratio, enabling a greater uptake of nutrients, an enhanced competitive ability under low nutrient regimes and the possibility that they are able to exist in more oligotrophic regions than diploid cells (Qiu et al., 2012). The strategy of viral resistance in the haploid phase is beneficial and deleterious alleles are not masked, so they can be purged from the population and successful new variants quickly fixed (Otto and Marks, 1996), enabling these populations to adapt more rapidly than those that are diploid (Zeyl et al., 2003). However, it may be that in other environments, the benefits of the haploid phase are outweighed by the benefits of recombination and meiosis in the diploid cells. We can conclude that the haploid stage of *E. huxleyi* may constitute a relatively persistent life stage and efforts need to become more focused on accounting for this in future research.

5. Coccoliths and calcification

Turning our attention back to the diploid cells of *E. huxleyi*, a large section of the remainder of this chapter relates to how calcification is affected by environmental parameters. We find that there is little additional information regarding cytological observations of calcification since Paasche (2002), however a wealth of genomic studies have attempted to understand the molecular mechanisms underlying this process. Additionally, attempts have continued to address whether calcification acts as a carbon concentrating mechanism. To introduce or perhaps refresh the reader, we will now briefly re-review cytology as a pre-requisite to addressing the chemistry and genomics associated with this biogeochemically important process before discussing calcification-photosynthesis interactions.

5.1. Cytology

The cellular mechanisms controlling the complex morphology of coccoliths and the variation in rates of production of $CaCO_3$ were a focus of attention for biologists and ecophysiologists over the last century, following the discovery of coccoliths by C.G. Ehrenberg (1795–1875) and their identification by T. Huxley and C. Emiliani. The cellular components involved in calcification in coccolithophores have been investigated mainly in *E. huxleyi*, as well as in other species such as *Coccolithus pelagicus* and *Pleurocrysis carterae* (Manton and Leedale, 1969; Van der Wal et al., 1985; Marsh, 1994; Taylor et al., 2007). Briefly, coccolithogenesis begins with the precipitation of $CaCO_3$ intracellularly, within the coccolith vesicle, a specialized compartment that seems to arise from merged membranes of Golgi origin in association with the nucleus (van der Wal et al., 1983). In some species (*E. huxleyi* and *Gephyrocapsa* sp.), a reticular body has been described associated with the distal side of the coccolith vesicle. These intracellular structures are also present in non-calcifying coccolithophore cells, apparently in deformed state and less closely associated with the nucleus (Klaveness and Paasche, 1971; van der Wal et al., 1983). The synthesis of coccoliths involves the formation of an organic scale or baseplate followed by nucleation of $CaCO_3$ crystals (Westbroek et al., 1989; Marsh, 1999; Young et al., 1999). The construction and mechanisms controlling $CaCO_3$ crystal formation appear to be highly dependent on calcium binding proteins (Corstjens et al., 1998; see section 4.5) and polysaccharides, which have been identified as tightly coupled with coccoliths (van der Wal et al., 1983; Marsh, 1994; Henriksen et al., 2004). Direct observations under the microscope have revealed that the formation of a coccolith is rapid, between 1 (Paasche, 1962) and 3.5 hrs (Taylor et al., 2007) and its secretion involves considerable contractile activity to eject and position the coccolith on the cell surface (Taylor et al., 2007).

5.2. Inorganic carbon and calcium kinetics

Calcification is the biogenic precipitation of $CaCO_3$ from Ca^{2+} and CO_3^{2-} precursors. Given that *E. huxleyi* precipitates $CaCO_3$ intracellularly, these cells require the uptake and delivery of Ca^{2+} and inorganic carbon to the site of $CaCO_3$ nucleation. Cells of *E. huxleyi* appear to import bicarbonate into the cytosol and it is thought that HCO_3^- ions are also imported into the coccolith vesicle, possibly by a Cl^-/HCO_3^- antiport mechanism (Holtz et al., 2013; see section 2.5 for a more thorough discussion of the potential transporters involved in calcification). Inside the coccolith vesicle and calcification fluid, the conversion of HCO_3^- into CO_3^{2-} is required in order to maintain a supply of inorganic carbon for $CaCO_3$ precipitation. In this process, H^+ are generated and these must be extruded in order to maintain elevated pH in the calcification fluid. A model proposing the activity of carbonic anhydrase (CA) inside the coccolithophore vesicle, a Ca^{2+}/H^+ exchanger, and a supply of DIC exclusively *via* CO_2 diffusion has been proposed as the most likely mechanism for calcification because CA and Ca^{2+}/H^+ exchangers have already been discovered in *E. huxleyi* (Holtz et al., 2013). Furthermore, this method is likely to be energetically advantageous given that the pH of the cytosol is significantly lower than that of the calcification fluid in *E. huxleyi* (Dixon et al., 1989; Nimer et al., 1994).

Many studies have focused on the mechanisms controlling the acquisition of substrates required for coccolith formation although perhaps fewer exist about the mineralogical characteristics of coccoliths. Mineralogical composition is important when trying to predict the thermodynamic stability and preservation of $CaCO_3$ based on not only its physiological controls, but also the chemical processes affecting dissolution. Indeed, it has been assumed that coccolithophores produce $CaCO_3$ only in the form of calcite; however, recent evidence suggests that coccoliths can also be made of aragonite or vaterite (Wilbur and Watabe, 1963), two polymorphs of $CaCO_3$ with substantially higher susceptibilities to dissolution (Morse et al., 2006). This work has been disputed by Young et al. (1991), but the two studies used

different strains suggesting that different subspecies/strains may display different mineralogies under varying environmental conditions.

5.3. Carbon sources in photosynthesis and biogeochemical implications

Neither photosynthesis nor calcification in *E. huxleyi* appears to be saturated at ambient concentrations of dissolved inorganic carbon (DIC) in seawater (Herfort et al., 2002). The form of DIC used by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is CO_2 (Cooper et al., 1969). However, the concentration of CO_2 in seawater is only ~1% of the total DIC compared to bicarbonate, with makes up ~90% of the DIC. To overcome this limitation, most photosynthetic organisms have developed a carbon concentrating mechanism (CCM), by which either CO_2 or HCO_3^- is transported actively across membranes. However, coccolithophores are considered to have generally inefficient CCMs (Badger et al., 1998; Reinfelder, 2011) and indeed, coccolithophores may be limited by CO_2 under certain conditions (Ratti et al., 2007; Riebesell et al., 2007; Reinfelder, 2011). Carbonic anhydrase (CA), which catalyzes the conversion of bicarbonate, CO_2 and water, has been measured in coccolithophores (Sikes and Wheeler, 1982; Rost et al., 2003) although its involvement in a CCM is still unclear. It appears that *E. huxleyi* acquires bicarbonate mainly by an anion exchange protein, but external CA can also be activated at low concentrations of DIC (Herfort et al., 2002). It has also been suggested that calcification can provide an additional source of CO_2 (Ware et al., 1992; Frankignoulle et al., 1994; Buitenhuis et al., 1999), since this it is produced during the calcification reaction. The net production of CO_2 is therefore a nonlinear function of $CaCO_3$ precipitation and carbon fixation *via* photosynthesis, and coccolithophores are likely neutral or represent a net sink for CO_2, except perhaps at very low concentrations of CO_2 (Reinfelder, 2011). The extent to which a calcifying organism represents a source or a sink of CO_2 to the environment is critically dependent on the production ratios of particulate inorganic carbon (PIC, often used interchangeably for $CaCO_3$) to particulate organic carbon (POC). Specifically, *E. huxleyi* represents a sink of CO_2 to the environment if the PIC:POC ratio is <1.5, but it is a source of CO_2 if the ratio is >1.5 (Frankignoulle et al., 1994). This

ratio in *E. huxleyi* has been found to vary from 0.2 to >1 (Riebesell et al., 2000; Zondervan et al., 2001, 2002; Engel et al., 2005; Feng et al., 2008; Iglesias-Rodriguez et al., 2008), indicating that *E. huxleyi* acts as a CO_2 reservoir.

5.4. Calcification/photosynthesis interactions

Several studies have investigated the possible physiological coupling between photosynthesis and calcification, although these feedbacks are still unclear. As discussed previously, the generation of CO_2 through calcification has been proposed as a possible CCM to increase the CO_2 availability at the site of RuBisCO, not only in coccolithophores (Buitenhuis et al., 1999) but also in other calcifying organisms including calcareous algae (Ries et al., 2010), and corals (Gattuso et al., 1999). In fact, although some calcifiers can accumulate sufficient DIC for photosynthesis in the absence of calcification, it has been suggested that, under nutrient- and light-replete conditions, calcification may partly support photosynthesis (Reinfelder et al., 2011). A general consensus has, however, not been reached and other studies have found that photosynthesis and calcification are decoupled in coccolithophores (Buitenhuis et al., 2001) and corals (Gattuso et al., 2000). Additionally, whilst calcification in *E. huxleyi* has been found to occur predominantly during the light phase (Paasche, 1962, 1964, 1965, 1966; Sikes et al., 1980; Linschooten et al., 1991) in some cases it can continue in the dark (Trimborn et al., 2007; Leonardos et al., 2009). Furthermore, in experiments using media lacking Ca^{2+} to prevent $CaCO_3$ precipitation, no effect was detected on organic carbon production suggesting that photosynthesis can be decoupled from calcification (Herfort et al., 2002.,Trimborn et al., 2007; Leonardos et al., 2009).

5.5. Genomics of calcification.

Almost nothing was known about the molecular biology underpinning the tightly controlled intracellular process of calcification when Paasche synthesized his review in 2002. The only potential protein identified was GPA, a highly acidic Ca^{2+} binding molecule named for its

high concentration of glutamic acid, proline and alanine residues (Cortstjens et al., 1998). However, the role of GPA is still putative and recent studies find conflicting results on its expression in relation to calcification (Quinn et al., 2006; von Dassow et al., 2009; Richier et al., 2009; Rokitta et al., 2011; Mackinder et al., 2011; Benner et al., 2013; Kegel et al., 2013).

Since 2002, a variety of genomic studies have attempted to look for proteins involved in biomineralization. Initial efforts involved growing cells under nitrate or phosphate limitation to stimulate calcification (see section 5.4) and identify differentially expressed transcripts using methods such as EST analysis (Wahlund et al., 2004), suppressive subtractive hybridization (Nguyen et al., 2005), SAGE (Dyhrman et al., 2006) and microarrays (Quinn et al., 2006). These studies showed that proteins such as carbonic anhydrase (Quinn et al., 2006) and calcium binding proteins (calnexin and calretulin, Wahlund et al., 2004) had potential roles in calcification, although results were difficult to interpret because of possible interactions with nitrate and phosphate starvation. More recent attempts have applied transcript-based techniques to the haploid and diploid phases of *E. huxleyi* (Richier et al., 2009; van Dassow et al., 2009; Rokitta et al., 2011; Mackinder et al., 2011) and were assisted by sequencing of the genome of strain CCMP1516 (Read et al., 2013), which enabled more extensive transcript annotation. Since only diploid cells calcify, the premise was that highly up-regulated transcripts from this phase could be involved in calcification. These studies have attempted to shed light on transporters that may have roles in providing Ca^{2+} and carbon substrate biomineralization (reviewed by MacKinder et al., 2010).

Despite best molecular efforts, still little is known about cellular Ca^{2+} transport dynamics in *E. huxleyi* and EST-based methods failed to find any Ca^{2+}-ATPases associated with calcified diploid cells (von Dassow et al., 2009). However, transcripts from a Ca^{2+}/H^{+} VCX1-type antiporter and NCKX proteins associated with rapid Ca^{2+} efflux (von Dassow et al., 2009), as well as CAX3 (a Ca^{2+}/H^{+} transporter, von Dassow et al., 2009; Mackinder et al., 2011; Rokitta et al., 2011) were found in diploid cells. The latter has been proposed to have a role during

transport of Ca^{2+} to the endomembrane system for calcification (Mackinder et al., 2011). The up-regulation of Solute Carrier 4 (SLC4) in diploid, calcified cells (also known as AEL1, a Cl^-/HCO_3^- transporter, von Dassow et al., 2009; Mackinder et al., 2011; Rokitta et al., 2011) suggested that this protein is involved in calcification related HCO_3^- dynamics, either enabling HCO_3^- to cross the plasma membrane or enter cellular compartments involved in biomineralization (von Dassow et al., 2009; Mackinder et al., 2011). Interestingly, RNA-seq analysis showed that none of these genese were differentially expressed in *E. huxleyi* cells that presented higher calcification rates under different CO_2 levels in long-term chemostats (>700 generations, Benner et al., 2013) and both CAX3 and NCKX2 were expressed at high levels in all treatments. The lack of a confirmed trend for carbon substrate or Ca^{2+} uptake means that it is plausible that many transporters involved in calcification are as yet undiscovered.

Cytological dynamics during biomineralization also remain relatively unknown for *E. huxleyi*. On the basis of proteomic and transcriptomic data, clathrin-mediated endocystosis/vesicular trafficking has also been proposed to be involved in calcification (Jones et al., 2011; Rokitta et al., 2011), whereas Quinn et al. (2006) suggested that α-soluble *N*-ethylmalemimide sensitive factor attachment (SNAP) proteins and RabGAP/TBC-containing proteins may have a role in SNARE-type secretory dynamics during coccolithogenesis. Additionally, von Dassow et al. (2009) and Rokitta et al. (2011) found SNARE-type homologs associated with calcifying diploid cells. Haploid/diploid studies indicated up-regulation of components of vacuolar type H^+-ATPases in calcified diploid cells (von Dassow et al., 2009; Mackinder et al., 2011; Rokitta et al., 2011), and comparative genome hybridization (CGH) of sixteen strains recently indicated that that up-regulation of 2 V-type protein ATPase proteolipid subunits was associated with calcification status (Kegel et al., 2013). Activity of these pumps was previously located to *Pleurochyrsis* coccolith vesicles (Araki and Gonzalez, 1998) and subsequently subunits were immunolocalized to this cellular component (Cortsjens et al., 2001). It has been proposed that V-ATPases are involved in alkalinizing vesicles to enable a

suitable pH for coccolithogenesis but recent RNA-seq investigations indicated that these components were not always associated with calcification (Benner et al., 2013). Recently, Mackinder et al. (2010) challenged the theory of alkalization by proposing that V-ATPases were involved in coccolith vesicle acidification, with an influx of H^+ enabling the transport of Ca^{2+} to the site of calcification through CAX3 transporters, which was dismissed by Holtz et al. (2013; see section 2.3). It is clear the genomics unpinning substrate transport and pH regulation in *E. huxleyi* require much more investigation.

Whilst most researchers have focused their attention on transporters, components associated with $CaCO_3$ during biomineralization remain understudied in *E. huxleyi*. It is likely that coccolith morphogenensis is controlled in part by the cytoskeleton, since the inhibition of microtubules and actin results in the formation of malformed coccoliths (Langer et al., 2010). The identification of proteins with similarities to those involved in biomineralization within other organisms (e.g. Nguyen et al., 2005) indicates that many recent studies still contain a wealth of information waiting to be gleaned. However, time and time again, exploratory molecular studies uncover a substantial number of unknown genes and proteins containing sequences that cannot be found in any current genomic database or unusual proteins with conserved motifs (e.g. Wahlund et al., 2004; Nguyen et al., 2005; Quinn et al., 2006; Benner et al., 2013; Jones et al., 2013). Data from these studies may be a useful starting point for future investigations into the molecular dynamics of calcification, for example it is possible that genes containing a Ca^{2+} binding domain and a Ca^{2+}/Mg^{2+} channel, exhibiting homology to the connective tissue glycoprotein fibrillin, are involved in coccolith biomineralization (Benner et al., 2013). Additionally, proteins involved in vitamin K2 biosynthesis were found within the *E. huxleyi* proteome (Jones et al., 2013), a promising target since vitamin K2 plays a role in the process of Ca^{2+} chelation and biomineralization in human osteoblasts (Akedo et al., 1992). It has also been suggested that chitinase and chinodextrinase have a role in the dynamics of chitin-like polysaccharides that could bind coccoliths to the organic extracellular surface of *E. huxleyi* cells (Benner et al., 2013). Additionally, CGH analysis indicated that a

long chain fatty acid CoA ligase, an arylsulfatase with a Ca^{2+} binding subunit, an uncharacterized oxidoreducatase, an activator of a 90kDa heat shocker protein ATPase and a kelch-like protein were associated with calcification (Kegel et al., 2013). It is plausible that some or all of these proteins could have roles in the process of biomineralization and these studies remain a wealth of information for gene hunting. However, in the absence of a viable transformation mechanism for *E. huxleyi*, function and confirmation of their role may remain speculative.

6. Ecophysiology and responses to environmental change

Since Paasche (2002), a substantial body of literature has appeared regarding the effect of environmental change and future ocean conditions on *E. huxleyi* growth and calcification. Specific responses in relation to temperature, salinity, nutrient availability and carbon chemistry will be reviewed in this section. Recent molecular studies regarding irradiance will be briefly described and for a thorough overview of the response of this species to light, particularly with respect to calcification, we direct the reader to Zondervan (2007). As previously stressed, general assumptions on physiology should be made with caution considering the vast levels of diversity that exist at the molecular level between strains (section 2). We find that in the years since the Paasche review, multi-parametric studies are becoming more common in the literature, yielding a more complete picture of how environmental dynamics affect *E. huxleyi* physiology.

6.1. Temperature

An extremely successful species, *E. huxleyi* has been observed globally from subtropical to subpolar waters. Recent observations indicate that the species may also be present at low densities in Southern Ocean waters below 60°S (e.g. Cubillos et al., 2007; Gravalosa et al., 2008) and in high Arctic waters (80°N; Heimdel, 1983; Hegseth and Sundfjord, 1998; Charalampopoulou et al., 2011). Satellite observations also indicate a poleward expansion of *E. huxleyi* between 1997-2007 (Winter et al., 2013). These findings challenge previous

paradigms regarding the distribution of this species that were described by Paasche in 2002 but the extent to which this relates to climate change, physiology, current dynamics or is the result of higher resolution sampling remains difficult to determine. However, the near uniform geographical distribution of this species suggests local populations exist adapted to different hydrographic regimes.

Temperature has been considered as a primary control factor setting the boundaries of *E. huxleyi* biome (Boyd et al., 2010) as well as morphotype distribution (Mohan et al., 2008; Henderiks et al. 2013; see section 2), and many studies have investigated how regional projections of warming might alter *E. huxleyi* physiological performance. As reviewed by Fielding (2013), the specific growth rate of *E. huxleyi* generally increases between 2ºC and 27ºC, with maximum growth rates equaling ~2 d^{-1}, although data are typically clone specific (Paasche, 2002). Although growth data do not exist for temperatures below 2ºC, the few studies conducted above 27ºC have showed growth rates close to zero (Fielding, 2013). Although most models on *E. huxleyi* bloom formation use an exponential relationship to define the relationship between the maximum growth rate and temperature, a meta-analysis indicated that a power function (μ_{max}= $0.1419T^{0.8151}$) seemed to be a more appropriate relationship to use in models incorporating temperature-dependent maximum growth rates of *E. huxleyi* (Fielding, 2013).

Emiliania huxleyi seems to show a variety of responses to temperature with respect to calcification, however no molecular studies have been conducted on the mechanisms behind this variability to date. A decrease in temperature from 20 to 10ºC enhanced cell protoplast and coccosphere size as well as coccolith production for strain EH2 (Sorrosa et al., 2005), suggesting that calcification might increase as growth rate decreases. Additionally, an increase in temperature from 13 to 17ºC decreased calcification rates and coccosphere size for strain AC481 with no change in coccolith morphology, suggesting a lower $CaCO_3$ content per coccolith, a decrease in coccolith number per cell and/or a decrease in coccolith production rate under high temperatures (de Bodt et al., 2010). However, an increase from 10 to 20ºC did

not affect coccolith size for the PLY B92/11 strain (Fielding et al., 2009), thus it is important to recognize that strains differ in their thermal optimum and physiological responses. Also, most physiological studies have focused on coastal strains (Fielding et al., 2013), isolated from waters with a wider thermal window, and therefore might demonstrate an enhanced ability to adapt to increasing water temperatures (Huertas et al., 2011).

6.2. Salinity

Reflecting its global distribution pattern, *E. huxleyi* is found at salinities ranging from 11 to 41‰ (Winter et al., 1994). Although salinity does not appear to be an important factor defining bloom formation of this species, it seems to have an effect on the regulation of calcification although the underlying mechanisms remain unclear. Recent morphometric analysis of globally distributed sediment core tops, plankton samples and culture experiments demonstrated a positive correlation between *E. huxleyi* coccolith size and sea surface salinities between 33 to 38‰ (Bollmann and Herrle, 2007; Bollmann et al., 2009; Fielding et al. 2009) suggesting coccolith morphology could be used as a proxy for paleo-surface salinity reconstructions. However, this linear relationship has only been resolved for open-ocean data and coccoliths from marginal environments (e.g. Norwegian coastal waters, Black Sea and Northern Red Sea) deviate from this correlation (Fielding et al 2009). This again reflects the possible variation in the adaptative capabilities of different *E. huxleyi* morphotypes and clones linked to biogeography and evolutionary history. In fact, as reviewed by Paasche (2002), laboratory studies revealed that coastal clones were generally less tolerant to salinity changes than coastal ones. Further analysis of *E. huxleyi* strains from both marginal and open-ocean settings are required to gain more insights into coccolith morphology and sea surface salinity relationships.

6.3. Irradiance

Growth rates of *E. huxleyi* are light dependent and typically increase as light level increases, until a certain limit when growth becomes saturated (see review by Zondervan, 2007). Light

is an important requirement for *E. huxleyi* bloom formation and blooms are primarily confined to the top 10-20 m of the water column, associated with high light conditions (e.g. Nanninga and Tyrrell, 1996). In contrast to other phytoplankton groups, *E. huxleyi* does not show photoinhibition at irradiances up to 2500 µmol photons m^2 s^{-1} (Zondervan. 2007), and saturation irradiances are higher than those for dinoflagellates or diatoms (as described by Paasche, 2002). These differences confer a competitive advantage to *E. huxleyi* in stratified waters,. Greater resilience to photoinhibition might also benefit *E. huxleyi* under future climate conditions, where mixed layer depths are expected to be shallower, trapping phytoplankton closer to the surface and exposing cells to increased light irradiances.

Recent molecular studies have revealed that photoacclimation in this species involves a trade-off between light absorption and photoprotective characteristics that might include changes in abundance and composition of proteins in the light-harvesting antenna of PSII (Lefebvre et al., 2010; McKew et al., 2013a, b). Additionally, only ~20% of the proteome is differentially regulated when cells were grown at 30 and 1000 µmol photons m^2 s^{-1} (McKew et al., 2013b), making it difficult to predict the success of *E. huxleyi* with our current understanding of mechanistic responses. These studies suggest that in intermittent high-light environments *E. huxley* maintains a stable core of catalysts involved in CO_2 fixation, respiratory energy metabolism and biosynthesis.

Dissimilarities between *E. huxleyi* and diatom pigment composition may explain differences in light harvesting efficiency and photoprotective abilities between these two groups (van de Poll et al., 2007). Calcification may also provide an additional advantage for *E. huxleyi*; sudden increases in light intensity produce an excess of energy that might be used to channel CO_2 towards organic carbon fixation (transport to the proximities of RuBisCO), and Ca^{2+} and inorganic carbon into the calcification vesicles. For example, an abrupt increase in light from 50 to 800 µmol quanta m^2 s^{-1} prompted a rapid (11-fold) increase in calcification rate by *E. huxleyi* in the first 10 minutes of light exposure, which was two-fold more than the increase in

the carbon fixation rate during the same period (Barcelos e Ramos et al., 2012). In contrast, cells of the diatom *Phaeodactylum tricornutum* used the extra energy to increase carbon fixation rates (Barcelos e Ramos et al., 2012). Although the role of coccolithophore calcification is still uncertain, this result might suggest a potential function in dissipating excess energy during cellular acclimation to new light conditions. The presence of coccoliths has also been proposed to serve as a light scatter mechanism to reduce cellular photo-damage at high irradiances (Gao et al., 2009).

Intraspecific differences in high light responses once again emphasize the complexity present within the *E. huxleyi* species group. For example, as light levels increase, most phytoplankton groups reduce the number of photosynthetic units (PSU) present per cell whilst keeping PSU size constant (Suggett et al., 2007). However, an increase in light levels caused the calcifying strain B92 to preferentially decrease PSU size instead of number whilst the non-calcifying strain (B11) decreased both PSU size and number to maintain a relatively constant balance of absorption energy between PSII and PSI under high light (Suggett et al., 2007). These responses are different to those exhibited by other microalgae and provide a basis to understanding the flexible acclimation mechanisms of *E. huxleyi* under high light. Additionally, the intraspecific variability in photoacclimation responses likely reflects genetic differences relating to evolutionary history and calcification status.

6.4. Macronutrient effects on physiology and ecology: phosphorus and nitrogen

Emiliania huxleyi is known to flourish after the demise of the diatom bloom, when nitrate, phosphate and silicate are limiting, and populations are predominantly found in stratified surface waters under conditions of moderate to low nutrient availabilities (Egge and Heimdal, 1994; Marañon et al., 1996; Litchman et al., 2006; Siegel et al., 2007; Leblanc et al., 2009). As reviewed by Paasche (2002), this ecological advantage that *E. huxleyi* possesses in oligotrophic waters may stem from low nutrient quotas, an extremely high affinity for

orthophosphate under phosphate-limiting conditions, and the expression of a constitutive an inductive alkaline phosphatase, which allows the acquisition of inorganic phosphorus from organic phosphorus pools. *E. huxleyi* is also able to better maintain photosystem II (PSII) function under nitrogen depletion compared to diatoms (Loebl et al., 2010). The species is a typically a poor competitor for ammonium and nitrate, however some strains are able to use a variety of dissolved organic nitrogen (DON) compounds such as amides (Palenik and Henson, 1997), or other organic nitrogen and phosphorus compounds (e.g. glycine, urea, glycerophosphate) that other coccolithophore species, such as *Coccolithus braarudii* and *Calcidiscus leptoporus*, cannot use (Benner et al., 2010).

Since Paasche (2002), both physiological and molecular studies have improved the knowledge about nitrogen and phosphorus preferences and regulation of macronutrient metabolism in *E. huxleyi*. Along with an increase in alkaline phosphate activity, three phosphate-regulated proteins (PRP1, PRP2 and PRP3) were detected by the biotinylation of cell-surface proteins in *E. huxleyi* under phosphate limiting conditions (Dyhrman and Palenik, 2003), and it was suggested that PRP3 might possess 5´-nucleotidase activity. Intraspecific differences in alkaline phosphatase activity and protein sequence may result in physiological differences between different strains under low phosphate conditions (Dyhrman and Palenik, 2003; Xu et al., 2006; Landry et al., 2009). SAGE analysis further identified potential genes and molecular pathways involved in nutrient scavenging by *E. huxleyi* and highlighted the potential importance of polyphosphate as a short-term storage pool for phosphorus (Dyhrman et al., 2006). Recent physiological studies have also revealed interesting mechanistic effects of phosphorus limitation. For example, under phosphorus scarcity, *E. huxleyi* was able to interchange phosphorus lipids for those that do not contain phosphorus, such as betaine lipids, a mechanism that reduces total cellular phosphorus demand by up to 30% (Van Mooy et al., 2009).

Although phosphorus metabolism has been fairly extensively studied in *E. huxleyi*, less is known about enzymes or transporters involved in nitrogen metabolism and its regulation.

Iwamoto and Shiraiwa (2003) characterized an *E. huxleyi* nitrate reductase (NR), which exhibited novelty in its subunit structure and kinetic properties when compared to NR sequences from other algae and plants. Landry et al. (2009) investigated the up-regulation of a putative amidase located on the cell surface, which appeared to be involved in the acquisition of nitrogen from amino acids under nitrate limiting conditions. This protein was expressed at the end of an *E. huxleyi* bloom in Norway and was suggested as an indicator of the nitrogen status in this species (Landry et al., 2009). Recently, Bruhn et al. (2010) investigated the expression of a putative formamidase (FMD), a glutamine synthetase (GS) and an NR when cells of *E. huxleyi* were grown under different nitrogen sources. Expression results indicated that *E. huxleyi* uses a transcript-related nitrogen preference hierarchy. When nitrate is abundant, low priority is given to the catabolism of organic nitrate into ammonium by FMD or the assimilation of nitrate into glutamine by GS (Bruhn et al., 2010). Recently, Kaffes et al. (2010) observed than when *E. huxleyi* was grown under concentrations of nitrate typical of the North Atlantic Ocean during the Spring (~10 µM), cells adjusted the kinetic paramenters of NR, nitrite reductase, GS and glutamate synthase to coordinate intracellular nitrate and CO_2 fluxes and thus maintain constant intracellular nitrogen and carbon ratios.

Interactions between calcification and ambient macronutrient concentration have been repeatedly observed in *E. huxleyi* and it is thought that calcification is enhanced by nutrient limitation. However, the mechanisms underlying the interplay between nutrient status and the transformation of inorganic carbon into $CaCO_3$ remain largely unknown. Paasche (2002) and Shiraiwa et al. (2003) suggested that *E. huxleyi* blooms in the ocean might be triggered by a supply of nutrients that stimulate organic matter production and cell growth, later leading to nutrient depletion suppressing growth and enhancing calcification. Müller et al. (2008) confirmed that calcification in *E. huxleyi* is tightly linked to the G1 phase of the cell cycle. During this phase, cells have a high nitrogen requirement in order to increase biomass and reach the size threshold for division. Consequently, nitrogen limitation results in a reduced cell size and a tendency for cells to remain in the G1 phase, resulting in an increase in cellular

$CaCO_3$ content. However, phosphorus limited cells were larger and more calcified since biomass buildup continued, but DNA synthesis and replication were likely inhibited, preventing cells from entering the S phase (Müller et al. 2008).

It is important to clarify that although $CaCO_3$ and POC cellular quotas vary under conditions of nutrient limitation, $CaCO_3$ and POC production might follow different patterns. In addition, differences are also observed between *E. huxleyi* strains and the distribution of *E. huxleyi* morphotypes may be partly dependent on the nutrient concentration in the ocean (Mohan et al., 2008; Henderiks et al., 2013), again highlighting the need for multiple strains and morphotypes in the design of laboratory studies.

6.5. Effect of inorganic carbon chemistry on Emiliania huxleyi physiology

Over the period known as the Anthropocene (Crutzen and Stoermer, 2000), as CO_2 of anthropogenic origin has been rising in the atmosphere, the concentration of CO_2 in seawater has increased proportionally as it has diffused into the ocean. Some of this dissolved CO_2 reacts with water to form carbonic acid (H_2CO_3), a process termed ocean acidification (Caldeira and Wickett, 2003, 2005). These chemical shifts have been found to have an effect on the acclimation of *E. huxleyi* such as modulation of its physiology (e.g., changes in photosynthesis, growth rates and calcification) in response to ocean acidification (e.g., Langer et al., 2009) and selection of morphotypes with distinct inherent calcification properties (e.g., Beaufort et al., 2011; Bach et al., 2012; Hendericks et al., 2012). Given the geographical extent and abundance of *E. huxleyi*, these shifts in cell physiology also have the potential to alter marine biogeochemical cycles.

Over the last decade, since the term ocean acidification was coined (Caldeira and Wickett, 2003), there has been a prolific generation of manuscripts dealing with the reciprocal interactions between the biology of coccolithophores and the changing inorganic carbon chemistry in the oceans. Much of this work concerns the regulation of calcification and stems

from our poor understanding of its biological and evolutionary roles. Indeed, this metabolic function is still under scrutiny in the fields of biology, geochemistry and genomics. Negative effects of ocean acidification on coccolithophores have been observed in lab and field studies but the responses appear to be species-specific and in some cases strain-specific (e.g., Iglesias-Rodriguez et al., 2008; Grelaud et al., 2009; Langer et al., 2009; de Bodt et al., 2010; Riebesell et al., 2000; Beaufort et al., 2011). Synthesis studies have confirmed a decline in calcification with exacerbated ocean acidification but with a great degree of sensitivities to increasing seawater CO_2 levels and high diversity of responses in all calcifying groups including coccolithophores (Kroeker et al., 2010).

Among the main concerns about the future of coccolithophores and other calcifiers is the extent to which the exceptionally fast alterations in carbon chemistry over the last 65 million years (Ridgwell and Schmidt, 2010) will affect the function and resilience of coccolithophores. Whereas the chemistry of ocean acidification is relatively well understood, much less is known about the biological and adaptive effects of these chemical changes. To date, the main gaps in our knowledge stem from the poorly known molecular/biochemical mechanistics of biomineralization and the challenges in deciphering biological calcium carbonate precipitation from abiotic dissolution driven by ocean acidification (Iglesias-Rodriguez, 2013).

It has been argued that the shifts in carbonate chemistry associated with ocean acidification demand energetically costly metabolic adjustments (e.g., Irie et al., 2010), often associated with some metabolic cost. Genome sequencing and long term lab manipulations are beginning to shed light into the extent to which *E. huxleyi* populations adapt over time (see section 6). Information at all levels of population organization, from genes to phenes at varying time scales, will enhance our ability to predict the fate of *E. huxleyi* in a high CO_2 environment.

7. Future directions

As we have discovered, discrepancies in calcification responses to simulated ocean acidification are probably just one manifestation of the intraspecific differences between *E. huxleyi* strains. As we move forward to think about how best to understand the physiological ecology of this important organism, we must also take into account that phenotypic and genomic properties of strains are known to vary over time under continuous culturing (Lakeman et al., 2009), which could cause identical strains to diverge between laboratories. Recently, some laboratories have addressed issues of phenotypic drift in long term experiments and have ensured that more realistic multi-parametric experiments are conducted to better help us understand responses of this important species to environmental perturbations.

7.1. Short-term versus long-term experiments

Emiliania huxleyi, along with many other phytoplankton species, has a short generation time and large population size, conferring the species with a high potential for acclimation and adaptation to environmental change (Elena and Lenski, 2003). Responses from short-term studies might, therefore, vary from those of long-term experiments since the latter allow for evolutionary responses and the selection of new clones or genotypes that might randomly arise as result of spontaneous mutation (Lohbeck et al., 2012). To date, most studies have investigated the response of *E. huxleyi* to different environmental parameters in short-term experiments. For example, only a few studies have tested the long-term responses of *E. huxleyi* to the single effect of CO_2 (>150 generations, Müller et al. 2010; >500 generations, Lohbeck et al, 2012), or the combined effect of CO_2 and nitrogen source (>200 generations, Lefebvre et al. 2012), or CO_2 and temperature (>700 generations, Benner et al. 2013). Both Müller et al. (2010) and Lohbeck et al. (2012) found a similar reduction in growth and calcification rate after short and long-term incubations. However, growth rates of cells grown

under high CO_2 for 500 generations were higher than growth rates of cells maintained at current-day CO_2 when both populations were briefly exposed to high CO_2,, indicating possible selection for genotypes with better fitness under high CO_2 (Lohbeck et al., 2012). Similarly, Benner et al. (2013) found that CCMP371 exhibited an increased inorganic carbon content and calcification rate under warm and acidified conditions after >700 generations, opposite to the effects of short-term exposure (Feng et al., 2008). These studies suggest that a deep-time perspective is needed to understand long-term effects of high CO_2 and help us estimate the speed and capacity of adaptation.

7.2. Multiparametric experiments

Laboratory studies assessing the effect of independent environmental parameters on the physiology of *E. huxleyi* are central to understanding the cellular mechanisms underlying physiological changes. However, reciprocal interactions between environmental parameters can yield different effects than individual variables alone, which can also limit the comparability of studies. When Paasche (2002) published his review, most studies had a uni-parametrical focus. However, there has been an increasing trend for multi-parametrical studies in recent years, especially those combining increasing pCO_2 with another variable (e.g. light, temperature, phosphorus or nitrogen availability). For instance, light may modulate the physiological effects of increasing CO_2 on growth and calcification of *E. huxleyi* (see Zondervan et al., 2007). Cellular calcification of strain CCMP371 were reduced under elevated CO_2 conditions only when irradiance was saturating (Feng et al., 2008). In contrast, a more pronounced decrease in $CaCO_3$ production was observed under low light compared to high light levels for the diploid strain RCC1217 when exposed to enhanced CO_2 concentrations (Rokitta and Rost, 2012). Studies have also focused on the combined effects of increased CO_2 levels and temperature. For instance, the sole increase in CO_2 had more of an adverse impact (alteration of coccolith morphology) on strain AC481 than just the increase of temperature or the combination of both factors (de Bodt et al., 2010). In contrast, a combined increase in elevated CO_2 or temperature drastically decreased $CaCO_3$ production and

enhanced *E. huxleyi* abundance in a natural North Atlantic Spring bloom, response that was not observed when those parameters were independently manipulated (Feng et al., 2009). The response of *E. huxleyi* to elevated CO_2 has been intensely studied in laboratory experiments under nutrient replete conditions (see section 5.5). However, despite the importance of nutrients on *E. huxleyi* growth and calcification and the projected extent of oligotrophic waters in future oceans, CO_2-related studies limiting nutrients such as phosphorus and nitrogen have not been considered until recently (Sciandra et al., 2003; Leonardos and Geider, 2005; Borchard and Engel, 2011, 2012; Müller et al., 2012; Lefebvre et al., 2012; Rouco et al., 2013). Coccolith production in these studies presented a different response to increasing CO_2 depending on the nutrient condition and the strain, although direct comparisons between strains are difficult to make since each study used different experimental approaches. In these studies, variations in growth and photosynthetic rate, coccolith morphology, organic carbon assimilation as well as cellular nutrient stoichiometry between treatments were also observed. These results are important since growth rates and the biochemical composition of phytoplankton influence grazing selection (Jones et al., 2002), and this has the potential to determine the prevalence of some strains *versus* others in future oceans. Recent studies also assessed the interactive effect of the nitrogen source (and enhanced CO_2 on carbon allocation, growth and morphology of CCMP371 (Lefebvre et al., 2012), observing that these two factors have a synergistic effect on *E. huxleyi* carbon partitioning. Studies such as this are necessary for an understanding of the response of *E. huxleyi* to future ocean conditions and for the parameterization of global biogeochemical models.

8. Conclusions: thoughts on how to move forward

In our updated review on the biology of *E. huxleyi*, we find that the community is at a crossroads regarding how to approach the vast levels of diversity that are beginning to be unveiled as modern sequencing technologies become widely embraced. We, along with other authors, find that a drastic re-evaluation of the species concept is required in marine

phytoplankton biology. Strain EH2 and CCMP1516 differ by 30.1%, whereas the genera *Gephyrocapsa* and *Emiliania* differ by only 30.4% (Kegel et al., 2013). Can we associate these differences with biogeochemistry? Challenges facing the next generation of coccolithophore biologists involve understanding whether this diversity matters and how it can be best represented in models. For example, *E. huxleyi* blooms occur under widely varying environmental conditions suggesting either that different environmental factors trigger their periodic dominance, or that blooms in different environmental regimes represent periodic dominance of individual species within the diverse *E. huxleyi* phylogenetic group. Unraveling the nature of these cause-and-effect dynamics will likely require synthesis of genetic, biochemical, and physiological information in future years. The power of sequencing approaches in the post-genomic era makes collection of some of these data relatively straightforward, given proper experimental design (see section 6). However, the anticipated promise of the sequencing revolution to resolve questions about algal ecophysiology is now recognized to depend on a more integrated understanding of genomics and physiological responses to environmental conditions.

As discussed, the recent genome comparison of fourteen *E. huxleyi* isolates demonstrated that this species is far more diverse than previously thought (Kegel et al., 2013, Read et al., 2013) - a discovery that has become a refrain of sorts in the field of microbial diversity. All phototrophs share basic metabolic units; including light harvesting pigments, components of the photosynthetic and respiratory electron transport chains, carbon fixation and catabolic pathways. Regulation of these metabolic units and their biochemical details can vary among algal groups and these differences are expected to lead to ecological niche differentiation. The genome sequence serves as a blueprint for the presence of the fundamental phototrophic metabolic units as well as unique features that underlie strain-specific biogeochemical roles. The goal is to identify these features, understand their environmental controls, and quantify their impact on growth. Although straightforward in principle, identification of these genomic features has proven to be challenging. In the case of *E. huxleyi*, 31% of the identified genes in

the CCMP1516 scaffold genome have unknown functions (961/30,569). Furthermore, the biochemistry needed to confirm ambiguous annotations is a known bottleneck in functional genomics.

There is a huge gap between the knowledge of a phototroph's genome sequence and predictions of carbon accumulation rates (primary production) under varying environmental conditions. Whole cell gene and protein analyses (e.g., transcriptomics and proteomics, respectively) can provide views of subcellular activity that bridge the gap between the genome sequence and expressions of cell growth. These analyses are crucial to establishing molecular linkages between the genome and metabolism. They can also reveal key regulatory switches that operate under different growth conditions and be used to identify molecular proxies for estimating process rates in the field. Further complicating interpretations of next-generation sequence data are reports indicating that the transcriptome and proteome are generally decoupled (reviewed in Nie et al., 2007), reflecting regulatory processes that act at the levels of both transcription and translation. Indeed, recent CGH analysis of 16 strains of *E. huxleyi* showed that only 11 genes were associated with calcification (Kegel et al., 2013) indicating the potential importance of post-transcriptional control on this biogeochemically important function.

The field of systems biology applies computational approaches to understanding how cells utilize the complete collection of metabolic pathways leading to cellular growth. Some of these computational models integrate genome sequence information and expressions of cell growth (typically pigment content and/or elemental stoichiometry) with the goal of calculating the optimal growth rate under varying growth conditions. Other models include trade-offs that may allow for cell persistence over increased growth rate (Geider et al. 2009). Such models of cell growth become more robust through the addition of constraints determined through next generation sequence technologies as well as by including multiple measurements of cell physiology (Figure 3). These constraints serve to limit the range of solutions available to optimize photosynthate distribution through the metabolic networks and

maximize growth rate (Follows and Dutkiewics, 2012). Flux-based analysis has emerged as a powerful computational tool that is built upon the foundation of a complete genomic sequence and can be expanded through additional layers of genetic expression of increasing complexity (e.g., transcriptomics and proteomics; Price et al. 2003). Such detailed computational approaches are likely needed to fully understand the processes underlying *E. huxleyi* growth dynamics, and they will undoubtedly reveal unexpected strategies for metabolic adaptation under certain growth conditions (e.g., Dyhrman et al., 2012).

Even more promising for understanding biogeochemical impacts of *E. huxleyi* is the opportunity to connect genomics and physiology to expressions of growth that are retrievable from space. We envision that these scales of study can be bridged by identifying key proxies of cellular status that can be linked to optical properties obtainable through remote sensing technologies. Thus, efforts to unravel the molecular biology controlling photosynthetic rates and primary production at the cellular level could merge with improved image analysis and interpretation of processes occurring at regional and global scales to yield meaningful understanding of *E. huxleyi* biogeography in an integrated, global context. We have come a long way since 2002 and as the community continues to uncover the secrets of this cosmopolitan species, we look forward to the next decade of *E. huxleyi* research.

9. Acknowledgements

We thank Jeremy Young and Alex Poulton for images, helpful suggestions and comments. We also thank Kristen Reifel for thoughts on an early draft of this chapter.

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Figure legends.

Figure 1.

MODIS image of a coccolithophore bloom taken over the Barents Sea in August 2007. The visible portion of the bloom covers approximately 150,000 square kilometers. Image credit: NASA Ocean Color.

Figure 2.

SEM images showing different morphotypes of *Emiliania huxleyi,* plate a) morphotype A coccolith (isolated from waters around Svalbard in 2010), b) morphotype A coccosphere (unknown origin), c) *E. huxleyi* var. corona (Canary Islands, Spain 1992), d) morphotype B coccosphere (Svalbard, 2010), e) type B/C coccosphere (Patagonian Shelf, 2008), f) type C coccosphere (Patagonian Shelf, 2008), g) morphotype O coccosphere (unknown origin), h) morphotype R coccolith (Svalbard, 2010), i) morphotype R coccosphere (strain NZEH, isolated from Bounty Bay, New Zealand, 1992). Image credits: b), c) and g) - Jeremy Young; f) and g) - Alex Poulton; remainder - Bethan Jones, unpublished.

Figure 3.

A systems biology approach to studying coccolithophore physiology. Controlled laboratory experiments to study phenotypic plasticity combined with genome and post-genomic sequence information provide the inputs and constraints for reconstructing metabolic networks. Computationally driven modeling is used to develop new hypotheses about metabolic mechanisms and predict growth responses to changing environments.

Chapter 5

Bioluminescent Microalgae by

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Bioluminescent microalgae

Martha Valiadi, Charlotte L. J. Marcinko, Christos M. Loukas, M. Debora Iglesias-Rodriguez

1. Abstract

Some phytoplankton species, specifically members of the dinoflagellates, possess the remarkable ability to produce bioluminescence. Many bioluminescent species are globally distributed (Lynch, 1978) and some form blooms, which may sometimes be harmful (Poupin et al., 1999; Valiadi & Iglesias-Rodriguez, 2013). The bioluminescence system of dinoflagellates is unique from a biochemical, cellular and evolutionary perspective. Regulatory aspects of bioluminescence in relation to cell physiology remain largely unknown, as does the ecological niche of light producing

organisms. Meanwhile, in the field, bioluminescence has been used to study ecological dynamics within the plankton and to monitor toxic blooms (Moline et al., 2009; Shulman et al., 2011; Le Tortorec et al., 2013). Studies on dinoflagellate bioluminescence have shed like on fundamental processes like cellular mechanotransduction (von Dassow & Latz, 2002; Chen et al., 2007; Jin et al., 2013), circadian rhythms (Hastings, 2009) and evolution of gene structure (Liu et al., 2004; Liu & Hastings, 2007). The molecular components of the reaction have been used as reporters in biomedical applications (Suzuki et al., 2005; Li et al., 2010) and whole cells have been used to visualize fluid flow (Rohr et al., 2002; Watanabe & Tanaka, 2011). Herein, we introduce key concepts and discuss current research that involves bioluminescent dinoflagellates. The research being conducted spans several different fundamental and applied fields, highlighting the vast benefits that dinoflagellate bioluminescence studies offer to biology and engineering.

2. General introduction

Dinoflagellates are the main eukaryotic protists that are capable of producing light (Widder, 2001; Haddock et al., 2010). Within this group, bioluminescence is present in a number of ecologically important and sometimes harmful species, many of which form blooms (Poupin et al., 1999; Valiadi et al., 2012). Indeed, dinoflagellates are responsible for most of the bioluminescence observed in the surface ocean (Tett, 1971), especially the globally known bright blue bioluminescent displays that have been reported since at least 500 BC (Harvey, 1957; Lynch, 1978).

The production of light occurs in dedicated organelles termed scintillons (DeSa & Hastings, 1968). Scintillons are dense vesicles approximately 0.5 – 0.9 µm in diameter (Johnson et al., 1985; Nicolas et al., 1987) and which, during the hours of darkness, are abundant in the periphery of the cell (**Figure 1**) (Fritz et al., 1990; Seo & Fritz, 2000). Scintillons contain the luciferin substrate, the

luciferase enzyme (LCF) and, in most species, a luciferin binding protein (LBP) (Schmitter et al., 1976; Johnson et al., 1985; Knaust et al., 1998; Akimoto et al., 2004). Luciferin, the substrate of the reaction is also present in the scintillons. It is thought to originate by the breakdown of chlorophyll in photosynthetic species (Nakamura et al., 1989; Topalov & Kishi, 2001), although evidence suggests that there is more than one precursor molecule for luciferin in bioluminescent dinoflagellates (Valiadi & Iglesias-Rodriguez, 2013). Light is primarily produced in response to mechanical stimulation due to shear stress (Maldonado & Latz, 2007), for example upon contact with grazers or by breaking waves (Latz et al., 2008). However, many dinoflagellates also flash or glow spontaneously at certain times of the night (Krasnow et al., 1980; Colepicolo et al., 1993; Latz & Lee, 1995).

The primary bioluminescent taxonomic order is the Gonyaulacales, although the ability to produce light is present in a few other phylogenetically disparate dinoflagellate taxa (Poupin et al., 1999; Valiadi et al., 2012). Several bioluminescent species are cosmopolitan in both coastal and open ocean regions and include important heterotrophs (e.g. *Noctiluca* and *Protoperidinium*) and toxic (e.g. *Alexandrium*) or generally harmful species (e.g. *Noctiluca*, *Lingulodinium, and Ceratium*). There is considerable variation within genera and species in the presence of bioluminescence. For example, while almost all *Alexandrium* species are bioluminescent, two key oceanic genera, *Ceratium* and *Protoperidinium*, contain a very low proportion of bioluminescent representatives (Valiadi et al., 2012).

3. Function of bioluminescence in dinoflagellates

For bioluminescence to persist it must confer some competitive advantage. The ecological advantage of a bioluminescent lure to attract prey in organisms such as deep sea angler fish is obvious. The advantage provided by bioluminescence in single celled organisms such as dinoflagellates is less so.

Wilson and Hastings (2013) hypothesized that bioluminescence systems initially evolved as oxygen detoxification systems in the early Earth and then acquired an ecological function later on, when other oxygen detoxification mechanisms appeared. In dinoflagellates, it is now generally accepted that bioluminescence can provide an advantage by acting as an ecological survival strategy.

One theory put forward by which bioluminescence provides a survival benefit to dinoflagellates is the 'burglar alarm' hypothesis. Burkenroad (1943) proposed "A peridinian sieved by a copepod might by its spark of light facilitate capture of the copepod by a herring… There might thus be a net advantage to the luminescent plankton from facilitation of the capture of primary predators…". Feeding activity by copepods on bioluminescent dinoflagellates is known to produce a bright spot of light lasting several seconds (Esaias & Curl, 1972) at a wavelength (~475 nm) which coincides with that required for maximum transmittance through water and the maximum visual sensitivity in many marine organisms (Tett & Kelly, 1973). Therefore, grazers feeding upon bioluminescent dinoflagellates are potentially highly visible to their predators.

The burglar alarm hypothesis has been supported by a number of laboratory studies which have indicated the ability of higher trophic organisms to utilise dinoflagellate bioluminescence to locate prey (Mesinger & Case, 1992; Fleisher & Case, 1995). Abrahams and Townsend (1993) found mortality rates of copepods increased by up to 70% in the presence of dinoflagellate bioluminescence and concluded that the observed increase in zooplankton mortality should translate into increased dinoflagellate survival. However, the study could not obtain cell density counts of dinoflagellates to support this conclusion and so could not unequivocally prove the survival benefit to the dinoflagellate population.

Bioluminescence may also provide an ecological advantage to dinoflagellates through a zooplankton 'startle response'. In this case, bioluminescent emission during close contact with a predator startles

the predator and causes it to change course (Buskey et al., 1983) or suspend its feeding activity providing the dinoflagellate cell time to escape (Esaias & Curl, 1972). Experimental studies have shown that in the presence of dinoflagellate bioluminescence the ingestion rates of copepods can be reduced by 30 to 70% (Esaias & Curl, 1972; White, 1979). Bioluminescent flashes can cause changes in copepod swimming characteristics, increasing the number of high-speed swimming bursts and decreasing the amount of slow-speed swimming characteristic of their grazing behaviour (Buskey et al., 1983; Buskey & Swift, 1985). It has been inferred that the increased high-speed swimming activity represents a startle response by copepods which leads to a reduction in grazing. It is important to note that the hypothesized ecological advantages of dinoflagellate bioluminescence are not mutually exclusive. Indeed, there may be multiple ecological mechanisms by which bioluminescence can provide a survival benefit to dinoflagellates.

4. Cellular signaling cascade from stimulation to flashing

The chemical reaction to produce light is pH-dependent as LCF and LBP, which binds luciferin at physiological pH become functional at ~ pH 6. At this pH structural changes in LCF make the luciferin binding sites available (Schultz et al., 2005) and LBP releases luciferin. Luciferin is oxidized by LCF to form oxyluciferin which results in the emission of photons (reviewed by Shimomura, 2006; Wilson & Hastings, 2013) in the form of a brief flash of blue light (with an approximate wavelength of 475 nm).

Translating a mechanical disturbance to a bioluminescence flash is achieved by a rapid a cascade of cellular processes (**Figure 2**). Shear stress exerted on the cell membrane triggers a mechanotransduction pathway involving GTP-binding protein coupled receptors in the plasma membrane (Chen et al., 2007). Increase in cytosolic Ca^{2+}, mainly by release from intracellular stores (von Dassow & Latz, 2002) generates an action potential across the vacuole membrane and the

associated scintillons. The action potential produced by these processes leads to an influx of protons from the acidic vacuole into the scintillons that protrude into the acidic vacuole, decreasing their internal pH from ~8 to ~6 (Fogel & Hastings, 1972). Voltage gated proton channels are involved in this process (Jin et al., 2013), presumably such as that recently characterized in another (non-bioluminescent) dinoflagellate (Smith et al., 2011). Recently, a role for stretch activated ion channels has also been revealed. These are activated by changes in the fluidity of the plasma membrane, for example by shear stress, which has now been shown to be the primary self-stimulator of spontaneous bioluminescence (Jin et al., 2013).

5. Molecular evolution

The evolution of dinoflagellate bioluminescence systems has been revealed through the sequencing and phylogenetic analyses of the dinoflagellate luciferase gene (*lcf*) and luciferin binding protein gene (*lbp*). The *lcf* in photosynthetic dinoflagellates is composed of three tandemly repeated domains (D1, D2 and D3) each consisting of a central region that is highly conserved at the amino acid level and encodes a catalytically active site (Li et al., 1997; Liu et al., 2004). This region is flanked by more variable N- and C-terminal regions with roles in the pH response and activity of the enzyme, respectively (Li et al., 2001; Suzuki-Ogoh et al., 2008). Four histidine residues in the N-terminal regions of each domain are thought to induce the pH-mediated conformational change that exposes the otherwise folded catalytic sites (Li et al., 2001; Schultz et al., 2005). An N-terminal gene region, which shows similarities to glutathione-S-transferase at the amino acid level, precedes the three catalytic domains in all studied species but has an unknown function (Li & Hastings, 1998; Liu et al., 2004).

Most photosynthetic dinoflagellates also contain *lbp*, which has now been fully or partially sequenced from five genera (Machabée et al., 1994; Hackett et al., 2005; Erdner & Anderson, 2006;

Uribe et al., 2008; Jaeckisch et al., 2011; Valiadi & Iglesias-Rodriguez, 2014). The *lbp* of *L. polyedrum* occurs as a gene family with two distinct variants with approximately 86% identity (Lee et al., 1993; Machabée et al., 1994), each composed of 4 repeat domains with little similarity between them, unlike the homologous domains *lcf* (Liu & Hastings, 2007). Both variants are expressed at equal level (Machabée et al., 1994). We recently amplified *lbp* sequences with similar features from another four photosynthetic genera (Valiadi & Iglesias-Rodriguez, 2014).

An intriguing feature of the bioluminescence related genes of photosynthetic species is their evolution. Sequence comparisons of individual domains from triple domain *lcf* genes of different species have shown that corresponding domains of *lcf* are more similar in different organisms (e.g. D1 of *A. tamarense* and D1 of *P. reticulatum*) than are different domains within each organism (e.g. D1 and D2 of *A. tamarense*) (Liu et al., 2004). This is likely to reflect an ancient triplication of a *lcf* domain that was then carried forward during the evolution of the different photosynthetic species (Liu et al., 2004). However, the group of photosynthetic dinoflagellates that has been studied comprises very closely related species and it is therefore likely that much more variation exists, even within photosynthetic species. For instance, a highly divergent *lcf* gene exists within *Ceratium digitatum* (Valiadi et al., 2012) which is a representative of a cosmopolitan open ocean bioluminescent dinoflagellate genus (Swift et al., 1995). Further divergence among sequences arises from the multiplications of gene copies in dinoflagellate genomes. Both *lcf* and *lbp* phylogenies have showed that the divergence of sequences between different gene copies may exceed the divergence of sequences from different organisms (Valiadi et al., 2012; Valiadi & Iglesias-Rodriguez, 2014). Therefore, both genes appear to be under strong selection pressure leading to rapid continuous mutations.

The complexity of the evolution of dinoflagellate bioluminescence system is highlighted by unique bioluminescence gene of *N. scintillans* (Liu & Hastings, 2007), a heterotrophic dinoflagellate which

is ancestral to photosynthetic bioluminescent species. Its *lcf* consists of only one domain which is shorter than that of photosynthetic species and it is attached to *lbp* as a single hybrid gene (**Figure 3**). The N-terminal gene region lacks any sequence similarity and is much shorter than that of photosynthetic species. Additionally, the *lcf* domain is shorter by 60 AA at the N-terminal than the *lcf* domains of photosynthetic species and is thus missing 3 of the 4 histidines that are thought to be responsible for pH regulation (Li et al., 2001), although the pH regulation remains. Despite these major structural differences, both the functionally relevant central part of the *lcf* domain and *lbp* domain retain sequence similarity at the amino acid level to *L. polyedrum*. A likely scenario is that the *N. scintillans lcf*/*lbp* could have undergone fission giving rise to the separate genes in photosynthetic species. The single *lcf* domain could have then triplicated in photosynthetic species to produce a protein with a triple catalytic capacity and hence a higher bioluminescence yield. Further evidence for such an evolutionary event is supported by a solitary *lbp* domain in *N. scintillans* which is not attached to *lcf*, but rather directly to the N-terminal gene region (Valiadi & Iglesias-Rodriguez, 2014). Now it is important to determine whether there is also a solitary *lcf* in *N. scintillans*, which could indicate fission in some of the *lcf*/*lbp* gene copies, or if the solitary *lbp* represents a splice variant of the original hybrid gene. Answering these questions is important to reveal the selective pressures that drive the evolution of bioluminescence through time. For example, gene fission in *N. scintillans* could arise from the need to differentially regulate the expression of *lcf* and *lbp*, allowing the enzyme to be recycled while the binding protein can remain proportional to the substrate pool. A triplication of *lcf* domains and therefore a triplication of catalytic capacity could signify the need to maintain bioluminescence intensity while reducing the number of scintillons that can be contained in these smaller cells (Valiadi & Iglesias-Rodriguez, 2013).

6. Physiology

6.1. Diet effects on bioluminescence

The relation of bioluminescence to cell metabolism and overall physiology has not been investigated much. In general, when cultures of photosynthetic species become nutrient depleted (Esaias et al., 1973; Sweeney, 1981) bioluminescence was diminished. A small scale study that directly assessed the bioluminescence response to changing nutrient concentrations found that reduced nitrate in the culture medium only affected growth of *L. polyedrum* but not bioluminescence per cell (Sweeney, 1981).In *Alexandrium fundyense* the expression of *lbp* is higher in phosphate limitation than in nitrate limitation (Erdner & Anderson, 2006). This indicates that more luciferin is produced under these conditions but it is not known whether bioluminescence capacity is affected in the same way. In heterotrophic organisms, bioluminescence may also diminish when cells are starved (Buskey et al., 1992; Buskey et al., 1994; Li et al., 1996) Additionally, combined effects are known in *Protoperidinium depressum* where the bioluminescence system became even more sensitive to photoinhibition, a phenomenon where light inhibits the mechanical stimulation of bioluminescence, after a period of starvation (Li et al., 1996). However, Latz and Jeong (1996) found that bioluminescence correlated with feeding frequency rather than growth rate in *P. divergens* and *P. crassipes* and that the cells continued to invest energy in bioluminescence despite being unable to grow.

Recently, the bioluminescence in the photosynthetic dinoflagellate *Lingulodinium polyedrum* has been studied in experiments imitating changes in seawater pH and carbonate chemistry expected under ocean acidification.

Laboratory studies using cultures of *Lingulodinium polyedrum* and *Ceratium fusus* have found significant positive relationships between organisms' bioluminescent capacity and previous daytime irradiance (Sweeney, 1981; Sullivan & Swift, 1995). Such responses signify photo-enhancement of dinoflagellate bioluminescence and indicate that the short term light history of organisms may be an important influence on night-time bioluminescence intensity. However, evidence for photo-

enhancement in natural dinoflagellate populations is mixed and not well defined due to lack of observations.

Within the subpolar North Atlantic (59° N, 21° W) during summer 1991, the bioluminescence capacity of *Ceratium fusus* showed no evidence of photo-enhancement. Comparisons indicated that cells from the dimly lit thermocline demonstrated a greater bioluminescence capacity than those within the well-lit mixed layer (Sullivan & Swift, 1995). Conversely, experiments conducted on natural mixed populations further south at the Porcupine Abyssal Plain (49° N 16° W) during summer 2009 indicated that exposure to daytime irradiance was required to maintain night-time bioluminescence and that maximum night-time bioluminescence intensity increased monotonically with previous daytime irradiance (Marcinko et al., 2013). These results suggest that the daytime irradiance received by the mixed bioluminescent populations had a direct influence upon the stimulated night-time bioluminescent intensity observed and support laboratory findings of photo-enhancement.

It is currently unclear whether photo-enhancement is restricted to specific bioluminescent species and the mechanism by which bioluminescence can be photo-enhanced is unknown. Further laboratory and field work is required to fully understand the effects of irradiance the dinoflagellate bioluminescence system, how common this is in natural populations and how it may vary spatially and seasonally.

7. Diel rhythm of bioluminescence

7.1. Studies of diel rhythm regulation in laboratory cultures

Most bioluminescent dinoflagellates display a diel rhythm in bioluminescence intensity, being much brighter in the night that in the day. The two main mechanisms underlying this variation are the

regulation of bioluminescence at the molecular and cellular level by a circadian clock and/or photoinhibition of bioluminescence in the day.

In photosynthetic dinoflagellates, bioluminescence exhibits a diel rhythm controlled by an endogenous circadian clock. This makes bioluminescence almost undetectable in the day and brightest at night when it is most effective. The regulation of bioluminescence circadian rhythms varies significantly in the two dinoflagellate species (*L. polyedrum* and *P. lunula*) that have been investigated in depth (reviewed extensively by Hastings, 2007). In *L. polyedrum* the quantity of LCF and LBP is regulated translationally, while mRNA levels of both genes remain stable throughout the light-dark cycle (Morse et al., 1989; Knaust et al., 1998; Mittag et al., 1998). It has been suggested that this process is mediated by a repressor protein which binds to the 3' untranslated region of the *lcf* mRNA (Mittag et al., 1994), however, this has been disputed (Lapointe & Morse, 2008). The LCF, LBP, luciferin and the scintillons themselves are destroyed at dawn and then begin to be resynthesized at dusk; they peak in quantity approximately four hours into the night when they reach amounts that are 10 times higher than in the day (Dunlap & Hastings, 1981; Johnson et al., 1984; Fritz et al., 1990; Akimoto et al., 2004). In contrast, *P. lunula* regulates its bioluminescence in a completely different way. The number of scintillons and their luciferin and LCF content, as well as *lcf* mRNA, do not vary (Knaust et al., 1998; Okamoto et al., 2001). Instead, the scintillons are relocated concurrently with the chloroplasts to modulate bioluminescence intensity, placing the scintillons at the periphery of the cell during the night but near the center of the cell during the day to prevent their stimulation (Seo & Fritz, 2000). These positional changes involved both actin filaments and microtubules of the cytoskeleton (Heimann et al., 2009). Recently, Hastings (2013) outlined a hypothesis for role of synthesis and destruction of proteins. He presented evidence indicating the re-allocation of amino acids in different proteins at different times of day in *L. polyedrum*. In this way, nitrogen can be conserved in an environment where it can be limiting (e.g. in a bloom). The lack of

destruction of protein in *Pyrocystis* can be justified by a rich nutrient supply in deeper water normally inhabited by this organism (Swift & Meunier, 1976). These findings are consistent with observations made by Roy *et al.* (2014) where cells of *L. polyedrum* that formed cold-induced cysts lowered their metabolic rate and arrested their bioluminescence circadian rhythm.

Bioluminescence intensity in most dinoflagellates is diminished in the day by photoinhibition (Sweeney et al., 1959; Esaias et al., 1973; Hamman et al., 1981b; Hamman & Seliger, 1982; Sullivan & Swift, 1994; Li et al., 1996). This is unrelated to whether or not a dinoflagellate also controls its bioluminescence by a circadian rhythm as it does not depend on the concentration of available substrate of protein for bioluminescence. Rather, the response to the mechanical stimulation is blocked, presumably by a change in the polarization of the cell membrane (Hamman & Seliger, 1982). In most bioluminescent species, maximal photoinhibition species is caused by blue light (Esaias et al., 1973; Hamman et al., 1981a; Sullivan & Swift, 1994; Li et al., 1996). The identity of the receptor of the inhibiting light is unknown, as are the cellular processes that bring about photoinhibition of bioluminescence. Nevertheless, limiting the energy expenditure associated with bioluminescence only to the times when it is visible is an important evolutionary adaptation.

7.2. *Diel bioluminescence rhythm in natural dinoflagellate populations*

Diel variations in bioluminescence potential have often been observed in field studies (**Figure 4**). Increases of several orders of magnitude between day and night have been recorded in many regions including the Southern California Bight, Black Sea and Vestfjord (Norway) (Greenblatt et al., 1984; Lapota et al., 1992a; Tokarev et al., 1999b). Measurements compiled across several regions of the world's oceans show night-time bioluminescent intensity in surface waters is typically 1 - 3 orders of magnitude greater than corresponding daytime intensities (Utyushev et al., 1999). Early explanations for this variability were attributed to changes in the composition of bioluminescent

organisms being measured (Greenblatt et al., 1984; Lapota & Losee, 1984) or the vertical migration of organisms through the water column (Seliger et al., 1962)(Seliger et al., 1962). However, variations are now known to be more often associated with endogenous circadian regulation or the exogenous diel rhythm of dinoflagellate bioluminescence.

Within the Sargasso and Mediterranean Seas diel was thought to be responsible for significant increases the number of flashes and intensity measured from day to night which could not be explained by changes in the vertical distribution of organisms (Batchelder et al., 1992; Cussatlegras et al., 2001). In Vestfjord, Norway, Lapota et al. (1992b) demonstrated that the diel changes in measured bioluminescence were caused by diel of naturally occurring *Ceratium fusus* and *Protoperidinium curtipes* which were found to increase their night-time flash intensities by 137 and 29 times respectively relative to daytime values.

A small number of field studies have detected circadian regulation of bioluminescence within natural dinoflagellate communities. These include within populations of *Pyrodinium bahamense* within Oyster Bay, Jamaica (Soli, 1966; Biggley et al., 1969) and mixed communities of bioluminescent species from coastal salt ponds in Woods Hole (Kelly & Katona, 1966). In Woods Hole detection of circadian regulation was not persistent year round and was more generally found to be associated with the seasonal presence of photosynthetic *Gonyaulax* spp. (Kelly, 1968; Yentsch & Laird, 1968). Similarly, evidence of circadian regulation in mixed dinoflagellate communities within the North Atlantic has also been attributed to the presence of photosynthetic *Gonyaulax* and *Pyrocystis* species (**Figure 5**, Marcinko et al., 2013). Differences in diel rhythms between species can make variations in bioluminescence within natural multi-species plankton communities complex and dependent on community structure (Utyushev et al., 1999).

8. Applications

8.1. Technological applications

Dinoflagellate bioluminescence and luciferase has recently been used in several applied sciences. The earliest technological use of bioluminescent dinoflagellates was the QwikLite toxicity assay which used several dinoflagellates as a reporter for toxic metals and organic pollutants in seawater (Lapota et al., 2007). Here, the diminishing of cellular bioluminescence was used to derive a toxicity index (LD50) to various compounds and samples of contaminated sediments, and compared with other ecotoxicological assays. Dinoflagellate bioluminescence was found to be highly sensitive and cost-effective approach to detect of toxicity in the marine environment. Dinoflagellate luciferase has now also been used as a reporter in biomedical research, especially in cell biology. Single domains of dinoflagellate LCF fused to genes of interest in mammalian cells produce fusion protein whose abundance can be monitored by a rapid bioluminescence assay, thus allowing for monitoring gene expression levels in the tagged genes (Suzuki et al., 2005). In a subsequent study, Kato et al. (2011) used dinoflagellate LCF to monitor the expression and kinetics of a cell membrane protein. The authors fused LCF to the extracellular part of platelet-derived growth factor receptor transmembrane protein of a mammalian cell, which could then be detected by the addition of luciferin. The extracellular part could be specifically monitored by adding luciferin which does not penetrate the cell membrane. The intracellular part could be also targeted separately by first quenching the extracellular part using sulfo-NHS-acetate and then lyzing the cells to expose the intracellular fraction to the added luciferin. Separate identification of cellular fractions allow for targeted studies on membrane trafficking and for testing the effects of pharmaceutical drugs. For example, Li et al (Li et al., 2010) used dinoflagellate luciferase to investigate treatments of beta-galactosidosis, a

condition leading to severe neuronal dysfunction in humans. Luciferase was used to monitor the degree of restoration of the enzyme activity of mutated beta-galactosidase after treatment with a chemical chaperone which stabilized the enzyme. High-throughput screening by means of a bioluminescence assay allows for effective testing of novel compounds for treatments in disease.

Another key use of dinoflagellate bioluminescence is in fluid mechanics. This has particular relevance for coastal engineering where accurate knowledge of a flow field guides the design of constructions like wave-breakers and erosion protection structures on beaches. The force field within fluids can typically only be visualized by contact on measuring devices, which themselves cause changes to the flow field. The incredible sensitivity of cells to shear stress and the magnitude of the light produced make them particularly useful as reporters of fluid forces without contact instrumentation. Two recent studies have explored the use of bioluminescent dinoflagellates to visualize and quantify the flow field associated with the flow of water of a rippled sea bed (Foti et al., 2010) and the impact of weights or water jets on water (Watanabe & Tanaka, 2011). These pilot studies showed that, although more work is needed to use dinoflagellates as quantitative reporters of flow, the approach shows great promise for directly measuring theoretically modeled flow fields. For example, Latz and Rohr (2013) recently took advantage of high speed imaging bioluminescent dinoflagellate cells to define the flow fields within bathyphotometers that differ in shape and stimulation strategy. These measurements are key for determining the optimal instrument design for accurate bioluminescence measurements in the ocean.

8.2. *In situ* Measurements and Field Ecology applications

A variety of instrumentation currently exists for making in situ measurements of planktonic flow stimulated bioluminescence. Instruments, known as bathyphotometers, generally comprise of a light-tight chamber containing a form of light sensor, such as a photo-diode or photomultiplier tube, into

which water is drawn at a known rate after being agitated to induce a bioluminescent response. A lack of standardization across instruments including differences in agitation methods and measurement approach (sampling frequency, calibration and processing) often makes the comparison of measurements from different studies difficulty (Bivens et al., 2002). However, a number of general patterns in the distribution of bioluminescence are known. Increased bioluminescence often coincides with areas of upwelling, such as that along the West African coast (Piontkovski et al., 1997), and at frontal zones (Lieberman et al., 1987; Cussatlegras et al., 2001). On a basin scale across the Atlantic, strong similarities have been found between the distribution of bioluminescence and chlorophyll-a (Haddock et al., 2010). The distribution of bioluminescence also varies with depth. Bioluminescence is typically highest within the mixed layer and decreases below the thermocline (Neilson et al., 1995) although exact profiles may change depending on location, water mass and time of year (Cussatlegras et al., 2001).

In situ measurement of bioluminescence has a number of applications for assessing the wider ocean environment. Specifically, it can be used to assess and monitor the structure and variability of marine ecosystems over a range of spatial and temporal scales (Kushnir et al., 1997). The spatial heterogeneity of bioluminescence can also be related to fronts, eddies and current meanders and consequently can be used to indicate the distribution of plankton biomass in relation to physical processes (Herring & Widder, 2001; Moline et al., 2007). Previous studies have used horizontal and vertical profiles of bioluminescence to identify internal wave motions (Kushnir et al., 1997), assess the small scale structure of plankton patchiness (Tokarev et al., 1998, 1999b) and the physiological state of planktonic ecosystems (Tokarev et al., 1999a). Bioluminescence has even been used to determine the affect of different seamount geomorphologies on the structure and distribution of the overlying pelagic communities (Tokarev et al., 2003).

Intensified cameras can be utilized to capture light stimulated from dinoflagellates as fish or other organisms move through the water (Clement et al., 2012). This allows the detection of fish schools at night from aircraft and their identification via the species specific patterns in bioluminescence they create (Squire & Krumboltz, 1981). Such methods have been used to evaluate stocks of anchovy, sardine, and tuna among other species across different regions including off the coast of Mexico and Chile (Squire & Krumboltz, 1981). Bioluminescence emissions can also be used in study the larger animals. For example, video imaging of light emissions from dinoflagellate has previously been employed to study the flow field around dolphins as they move through the water (Rohr et al., 1998).

One growing application of measuring planktonic bioluminescence is for the detection and monitoring of Harmful Algal blooms (HAB) in near shore waters. Dinoflagellates are estimated to account for approximately 75% of HAB species (Smayda, 1997). Many bioluminescent dinoflagellates produce harmful toxins, such as *Alexandrium tamarense,* or red tides, such as *Noctilluca scintillans,* which can have a disastrous impact on local environments and aquaculture (Anderson et al., 2012). Increased bioluminescence has been observed to coincide with red tides in the Southern Sea of Korea, even through the species responsible for the red-tide was not bioluminescent (Kim et al., 2006). Time-series monitoring within the Finish Archipelago Sea has also identified bioluminescence can be used to detect the onset of toxic blooms of *Alexandrium ostenfeldii* and study their spatiotemporal dynamics (Le Tortorec et al., 2013). Bioluminescence has the potential to provide a rapid biological marker for the presence of problem dinoflagellate species and could be used, in future, as part of an early warning system for harmful algal blooms in coastal regions.

Figure legends

Figure 1. Cells of *Pyrocystis fusiformis* from a clonal stationary phase culture in the dark phase. Cells were visualized under an epifluorescence microscope at 10x magnification. The luciferin in the scintillons emits blue fluorescence when illuminated with ultraviolet light. In this picture individual scintillons appear as white speckles within the cells. Most are localized at the cell periphery and some are within cytoplasmic strands which extend from the periphery, through the vacuole and towards the nucleus.

Figure 2. Schematic representation of part of a dinoflagellate cell depicting the cellular processes that take place to generate a bioluminescence flash. (1) Initially, a force is exerted on the outer cell membrane. Transduction of the signal from the outside to the inside of the cell involves the activation of a G-protein coupled receptor (Chen et al., 2007). The typical structure of such a receptor comprises seven transmembrane domains and the G-protein on the inner side of the cell membrane. The chemical signal is then transformed to an electrical signal on the vacuole membrane. (2) This is achieved by increasing the concentration of Ca^{2+} in the cytoplasm, mainly obtained from reserves within the cell and to a lesser extent from the external medium (von Dassow & Latz, 2002). This flux of Ca^{2+} causes the vacuole membrane to depolarize and generate an action potential. In this illustration, one of the scintillons protrudes into the vacuole while remaining attached to the vacuole membrane. This enables the action potential to travel to the scintillon membrane. (3) Voltage gated proton channels, which are presumably concentrated on the membrane of the scintillon rather than on the remaining vacuole membrane, are activated by the action potential allowing an influx of protons from the acidic vacuole. (4) This rapidly lowers the pH of the scintillons, activating the bioluminescent chemistry, and nearly instantly producing a flash of blue light. All the above processes, from the initial stimulus to the production of light, take place in just 20 milliseconds.

Figure 3. A representation of the different LCF and LBP structures, per cent identities between gene domains and the proposed scenarios for their evolution. *Lingulodinium polyedrum* (Lp) LCF is represents of all LCF's fully characterized from photosynthetic dinoflagellates to date(Liu et al., 2004). Lp LCF contains the three non-identical repeated domains, each 377 AA long with 150 AA in the central regions that encode the catalytic sites (patterned) and retain higher sequence similarity. The three domains are preceded by an N-terminal gene region. *L. polyedrum* also contains an LBP, which consist of 4 repeated domains and a similar N-terminal gene region to Lp LCF. In *Noctiluca scintillans* (Ns), LCF and LBP exist as a single gene. It contains only one LCF domain which is most similar to the second domain of Lp LCF. The LBP domain in Ns is structurally equivalent to that in Lp. The N-terminal gene region preceding the LCF and LBP in Ns is not structurally equivalent to that which precedes the analog genes in Lp. Two potential scenarios of how these gene structures arose are shown. In the gene fusion scenario (dashed lines), the second domain of LCF was excised (e.g. by splicing) and fused with LBP in *N. scintillans*. It is currently impossible to tell whether the solitary LBP in *N. scintillans* preceded the domain fused to LCF, or vice versa (dotted line). In the gene fission scenario (solid line), the Ns gene split and the LCF domain underwent successive duplications. Again, whether the Lp LBP could be derived from the fused LBP domain or the solitary LBP gene, is unkown.

Figure 4. Example of bioluminescence potential stimulated from a mixed community of

bioluminescent dinoflagellates collected from 5 m depth at the Porcupine Abyssal Plain observatory

in the North Atlantic. Samples of 2L volume were measured using a GLOWtracka bathyphotometer adapted for bench-top use (see Marcinko et al. 2013 for details). Bioluminescence was stimulated via flow through a 1 mm mesh excitation grid. Measurements were taken at a) night (23:00 hours) and b) during the day (08:00 hours).

Figure 5. Example of diel variability in bioluminescence potential stimulated from a community of bioluminescent dinoflagellates dominated by photosynthetic species including Gonyaulax and Pyrocystis species. One hundred and twenty litres of sea water was collected from 5 m depth at the Porcupine Abyssal Plain observatory in the North Atlantic during August 2009. Samples were incubated in situ, at a 16°C, and exposed to the natural photoperiod. A volume of 2L was measured hourly using a GLOWtracka bathyphotometer adapted for bench-top use and bioluminescence was stimulated via flow through a 1 mm mesh excitation grid. (Adapted from Marcinko et al 2013).

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Chapter 6

Harmful Algal Blooms and Microalgae: A European Perspective

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Harmful Algal Blooms and Marine Microalgae: A European Perspective

Johanne Arff and Belén Martín Míguez

1. Abstract

Please add 100-200 words abstract

2. Introduction

Algal blooms are a natural phenomenon being an important part of the marine food web and thus the basis for marine production and the exploitation of marine resources as a food provider. However some algal blooms are considered to be harmful to humans, marine mammals, birds, fish or the marine ecosystem as a total either due to effects from high biomass or production of biotoxins.

Mussels, scallops and clams are filter feeders, i.e. feeding upon microalgae, and some microalgae produce natural toxins that are not harmful to the shellfish itself or predators feeding on the shellfish. However, due to bio-accumulation these toxins impose a risk towards humans consuming contaminated seafood. On a yearly basis, almost 2000 cases of food poisoning from consumption of contaminated fish and shellfish are reported worldwide with about 15 percent having a fatal outcome (Hallegraeff 2003).

3. Harmful Marine Microalgae

3.1. Diatoms

Diatoms are one of the largest algal groups known, considered to encompass 10,000 to 12,000 species (Fryxell and Hasle 2004). Recently Mann and Vanormelingen (2013) have estimated the number of diatom species to be at least 30.000, and they suggest as much as a diversity of 100.000 different diatoms as a probable number of species. Diatoms are an ecological important algal group with an estimated contribution of 40-45 % of the oceans primary production (Mann 1999).

Any bloom of diatoms may be considered as potentially harmful due to ecological effects such as anoxic water, production of oil or mucus, bitter taste in shellfish or toxin production (Fryxell and Hasle 2004). Even if there is a vast number of diatoms there are at present only 14 known toxin producing species (Moestrup et al. 2013) constricted to the genera *Pseudo-nitzschia* (12 species), *Halamphora* (1 specie; syn. *Amphora*) and *Nitzschia* (1 species) all pennate diatoms; summarized in

Table 1. All toxic diatoms are identified as producers of the neurotoxin domoic acid (DA); the toxin responsible for Amnesic Shellfish Poisoning (ASP). The first incident of ASP was reported from Canada in November 1987 with more than 100 cases of illness, three having a fatal outcome, after consumption of toxic mussels. DA was detected (ref) and later *Ps. multiseries* was identified as the source organism (Bates 1989). This incident lead to an increasing concern regarding ASP outbreaks and several countries included DA and *Pseudo-nitzschia* in their monitoring programs on seafood safety resulting in the identification of other DA producing *Pseudo-nitzschia* species. DA is also recognized as the causative of mortalities of seabirds and sea mammals in California and Mexico feeding on contaminated anchovies or mackerel; *Ps. australis* being the source organism in all cases (Lefebrve and Robertson 2010). Accumulation of DA in different shellfish species was studied after a DA outbreak in Irish waters in 1999 (James et al. 2005); this study demonstrated that king scallops (*Pecten maximus*) are a more potent vector than blue mussels (*Mytlius edulis*), oysters (*Crassostrea edulis*) and razor clams (*Ensis siliqua*). Moreover DA accumulates differently in different tissue in *P. maximus* with highest concentrations and well above regulatory limit of 20 µg DA/g in hepatopancreas; and values below the regulatory limit in the abductor muscle (James et al. 2005). There are several studies on *Pseudo-nitzschia* spp. and DA in European waters; especially with respect to nutrients as a bloom regulator and spatial distribution of DA in bivalva. Bogan et al. (2007a, 2007b) demonstrated that the DA content in scallops from different harvesting grounds within relatively limited geographical areas (i.e. around Isle of Man and southern parts of Ireland) differed significantly, hence posing a risk with respect to the design of monitoring programs and the regulation of harvesting areas. Furthermore they discussed how different oceanographic conditions influencing the nutrient dynamics might explain the observed spatial differences of DA in shellfish. Another interesting feature with *Pseudo-nitzschia* spp. that may explain why higher DA values are detected in scallops than in other shellfish are the accumulation in thin layers, sometimes in depths below 10 m (Rines et al. 2010) which is well below the depths where mussels, oysters and clams are produced or harvested from, while scallops can have a vertical distribution as deep as 100 m (Hayward et al. 1996) and as such are more exposed for *Pseudo-nitzschia.* Furthermore, the sedimentation of *Pseudo-nitzschia* out of the productive zone might be another mechanism explaining the higher concentrations of DA in scallops

than in other shellfish. However, to the authors' knowledge the European monitoring programs on *Pseudo-nitzschia* and DA have been successful with no reports on human intoxications due to the consumption of DA contaminated shellfish so far in Europe.

There are several reports on mass occurrences of non-toxic diatoms and fish mortality; most of them related to aquaculture (i.e. caged fish are unable to escape unfavourable environmental conditions). Typically diatoms harm the fish by either clogging or damaging the gills, thus reducing the oxygen uptake through the gills. As a reaction to the diatoms the gill tissue produce mucus, hence the mortality is not caused by the diatoms itself but as a result of reduced oxygen uptake (hypoxia) and carbon dioxide excretion (hypercapnia) through the gill tissue (Rensel 1993). Species belonging to the genera *Cerataulina, Chaetoceros, Corethron, Leptocylindrus, Rhizosolenia, Skeletonema, Thalassiosira* have induced mortality in farmed fish (Smayda 2006). Especially the large *Chaetoceros* belonging to the subgenus *Phaeoceros* have protruding spines on their setae which can loosen from the algal cell and penetrate the gills resulting in bleeding. As refered by Smayda 2006 Chilean aquaculture have had losses due to blooms of *Chaetoceros* and *Leptocylindrus;* while *Corethron* and *Chaetoceros* have killed cultured salmonides in Canada (Yang and Albright 1994; Speare et al. 1989). *Chaetoceros* spp., *Skeletonema* and *Thalassiosira* spp. have been reported as the causative of mortalities in Scottish fish farms (Treasurer et al. 2003; Kent et al. 1995). Smayda (2006) also refers to two episodes in New Zealand where *Cerataulina pelagica* has killed fish by clogging the gills, as well as being an effect of post-bloom anoxia.

Furthermore, there are also reports on diatoms impacting European fisheries. In 1977 *Coscinodiscus wailesii* was first recorded in European waters; soon after local fishermen reported on excessive mucus clogging the gear (Boalch 1987). Moreover, Boalch reports that *C. wailesii* was demonstrated to produce mucus in laboratory cultures, and *C. wailesii* was recorded in samples collected in areas affected by mucus. This problem only lasted a few years, and Boalch suggested that this might be attributed to the observation of a change in dominating season for *C. wailesii;* i.e. from summer to winter and spring plankton. According to Smayda (2006) *C. wailesii* has also been a problem for

Japanese nori-production; competing for nutrients and hence reducing the yield. Smayda 2006 also refers to bird mortality in connection with a bloom of *C. concinnus* in the North Sea.

3.2. Dinoflagellates

According to Gómez (2012) there are 2377 described dinoflagellate species belonging to 259 genera, and of these are nearly 80 species listed as toxin producing species by Moestrup et al. 2013. The majority of known toxin producing microalgae belong to the dinoflagellates and until the early 1980s only dinoflagellates were identified as HAB species. Dinoflagellates produce a wide range of neurotoxins being responsible for ciguatera fish poisoning (CFP), diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP) and other harmful events such as allergic reactions and respirational irritation in humans.

Ciguatera fish poisoning – benthic species; dinophysis – okadaic acid, dinophysistoxins pectenotoxin, ; alexandrium – stx, spirolide; karenia/karlodinium/ med flere...

Saxitoxin (STX) and analogs are responsible for paralytic shellfish poisoning (ref), with *Gymnodinium catenatum, Pyrodinium bahmanese* var. *compressum* and *Alexandrium* spp. as identified source organisms (Anderson et al. 1989;). *G. catenatum* is the only STX-producing athekat dinoflagellate; and has a wide distribution with records in Europe, North and South America, Asia and Austrailasia (Guiry & Guiry 2013). *P. bahmanese* var. *compressum* is tropical ? *Alexandrium* is distributed all over the world with 33 recognized species (Balech 1995; Mackenzie & Todd 2002; Nguyen & Larsen 2004; Montresor et al. 2004) dived into two subgenera *Alexandrium* and *Gessnerium.* All toxin producing *Alexandrium* belong to the subgenus *Alexandrium* with 14 species listed in Moestrup et al. 2013; and *A. catenella, A. fundyense* and *A. tamarense* being responsible for the majority of PSP outbreaks on a global basis (ref).

In Ireland, azaspiracids (AZAs) is of major concern for the shellfish industry, with the first problems occurring in 1995 with human intoxications after consumption of toxic mussels produced in Irish waters (James et al 2002a). AZAs were also detected in shellfish from other parts of Europe; i.e. Norway, England, France and Spain (James et al. 2002b; Magdalena et al. 2003). Furthermore, edible

crab from Scandinavian waters has been shown to act as a vector for AZA (Torgersen et al. 2008). *Protoperidinium crassipes* was first pinpointed as the source organism (James et al. 2003), but recently the small dinoflagellate *Azadinium spinosum* was isolated during a research survey in the North Sea and shown to produce AZA (Krock et al 2009; Tillmann et al. 2009). Further work has resulted in the identification of several species belonging to *Azadinium*, with reports from South America (Hernandez-Becerril et al. 2012; Akselman and Negri 2012). At present only *Azadinium spinosum* and *A. poporum* (Tillmann et al 2011) are demonstrated to produce AZAs. Recently, Jauffrais et al. 2012 were able to demonstrate through feeding experiments that blue mussels fed with toxic *A. spinosum* accumulated AZAs above the regulatory limit of 160 µg kg^{-1}.

In Japan in 1975 and 1980 food poisoning after consumption of the bivalva *Pinna pectinata* affected more than 1700 and 900 persons, respectively (see Uemura et al. 1995 and references therein). Uemura et al. (1995) and Chou et al. (1996) were the first to isolate and describe pinnatoxins, a new neurotoxin. Even if pinnatoxins have been known for several decades the causative species was unknown until recently. Rhodes et al. 2010 were able to demonstrate that a cyst-forming peridinoide dinoflagellate isolated from the waters of New Zealand is a producer of pinnatoxin. Nézan and Chomérat 2011, however, were the first to describe a novel dinoflagellate *Vulcanodinium rugosum* that later was identified as the producer of pinnatoxins in Australia, New Zealand, Japan and China (Smith et al. 2011; Rhodes et al. 2011a; Rhodes et al 2011b). Pinnatoxins have also been detected in shellfish from Norway (Rundberget et al. 2010), Canada (McCarron et al. 2012), and France (Hess et al. 2013; Geiger et al. 2013). There are still uncertainties on pinnatoxins and acute toxicity towards humans (McNabb et al. 2012; Munday et al. 2012); hence further research is necessary to elucidate pinnatoxins as a food safety issue and the need for future regulations.

3.3. Haptophytes

Haptophytes, or prymnesiophytes, are known from both marine and freshwater systems. According to Anderson 1992 there is about 500 recognized species of haptophyta, and an estimated number of

species are 2000. Haptophytes have two flagella and are named after the haptonema, an appendage used for feeding or attachment. The haptonema can be short and stiff or long and coiling, and in some species it is rudimentary. All haptophytes have organic or calcareous scales, the latter belonging to the coccolithophorides, of which the scale structures have been important for the classification into species. The taxonomy of haptophytes was recently revised by Edvardsen et al. (2011) with introduction of new genus and transferring of species between genera.

Blooms of haptophytes can be conspicuous due to the shear biomass, i.e. recognised by either discolouration (coccolithophorides) or foaming and a gelatinous appearance (*Phaeocystis*). The scales of coccolithophorides scatter light, and during bloom situations the sea commonly is reported as turquoise. Moreover, the coccolithophoride *Emiliania huxleyi* forms massive blooms in the northern Atlantic during summers; the geographic distribution is easily recognised on satellite images (Figure 1).

Species belonging to *Prymnesium* are associated with fish mortality all over the world, and the first recorded fish kill caused by *Prymesium* was reported from the Netherlands in 1920 (see Moestrup 1994 for references). Moestrup also refers to episodes of fish mortality along the Baltic coast as far back as in the 1890s that later have been set in connection with *P. parvum.* He also mentions that fish kills caused by *Prymnesium* species have been reported from several European countries, i.e. Denmark, United Kingdom, Finland, Bulgaria and Norway, during the past century. In 2004 there was an extensive bloom of *Prymnesium* in Lake Koronia, Greece, with not only reports on fish mortality, but also bird kills (Michaloudi et al. 2009). According to the authors this is the first recorded death of birds due to *P. parvum.* However, Lindholm et al. 1999 reported that dead gulls had been observed by locals during the *P. parvum* bloom in Lake Vargsundet , Finland, in 1997.). Zingone et al. 2006 refer to an event with fish mortality due to *P. pateliferum*, now *P. parvum* f. *pateliferum* (Larsen 1999), from Italy. *P. parvum* blooms and fish kills have also been reported from Hungarian fish ponds (Vasas et al. 2012 Haemolytic toxins, prymnesins, are isolated from *P. parvum* and are identified as the toxic principle inducing fish mortality.

The bloom of *Prymnesium polylepis* (syn. *Chrysochromulina polylepis,* Edvardsen et al 2011) in Kattegat and Skagerrak in 1988 has been thoroughly studied throughout the years, and in this paragraph we rely on Smaydas (2006) review of the extensive research material. The first indications on a HAB came from the Gullmar Fjord, Sweden, with reports on abnormal behaviour and mortality of farmed fish. When the bloom reached Norwegian waters killing 900 tonnes of farmed salmonides the negative effects of *P. polylepis* became evident. Towing of fish-farms from the affected coastal areas into fjords with lower salinity turned out to be a successful mitigating activity reducing the amount of fish that actually could have been killed during the bloom. Another but not least important feature was the effects the bloom had on the ecosystem as a whole, with reports on mortality, not only in fish farms, but throughout the entire food chain including phytoplankton, zooplankton, benthic macroalgae and fauna, and demersal and pelagic fish. Professor Yasumoto and colleagues have demonstrated that *P. polylepis* produce two haemolytic and ichthytoxic compounds (see Moestrup and Thomsen 2004). The wide-range consequences of this bloom w in many ways a game changer increasing the awareness of HABs, as well as opening up for the establishment of regular algal monitoring programs.

A few years later, in May in 1991, a bloom of *Chrysochromulina leadbeateri* caused mortality of farmed fish in the Vestfjorden – Lofoten area in Northern Norway (). This was the first recorded bloom of this species, and as for *P. parvum* and *P. polylepis* there is evidence that the toxicity of *C. leadbeateri* is haemolytic compounds. In May 2008 unusual fish mortality was once again reported from the Lofoten area, and *C.* cf. *leadbeateri* was identified in samples collected from the affected localities (Arff and Tangen 2009). Furthermore, Smayda also refers to mortality of rainbow trout in connection with a bloom of *Chrysochromulina brevifilum, C. ericina, C. hirta,* and *C. spinifera* in Danish waters in 1992. However, there was no clear evidence on the harmful principles of these four *Chrysochromulina* species none being demonstrated as toxin producers. *C. leadbeateri* is also reported from the Mediterranean (Zingone et al. 2006).

From European waters the colonial stage of *P. globosa* and *P. pouchetii* are reported to form nuisance blooms. Blooms of *P. globosa* are a well-known phenomenon off the coasts of the Netherlands and

Germany where characteristic foam produced by the algae assembles on beaches. In Germany this algae is called "schaumalge" literally meaning "foam algae". *P. pouchetii* is common during the spring bloom of diatoms in Norwegian waters (www. Algeinfo.no), and especially in Mid- and Northern Norway. Moestrup (1994) refers to episodes with fish kills in Norwegian waters in connection with toxic blooms of *P. pouchetii,* but at that time *Phaeocystis* was only known to produce antibacterial acrylic acid. Tangen 1999, however, reports that the fish kills caused by *P. pouchetii* in Norway not are considered to be caused by toxins, but as an effect of the mucilage irritating and clogging the gills. More recent both *P. globosa* and *P. pouchetii* are reported to produce compounds with haemolytic activity, but the ichtyhotoxic mechanisms differ with *P. globosa* being a glycolipid producer, while there still are some uncertainties with respect to *P. pouchetii* (Rijssel et al. 2007).

Moreover blooms of haptophytes have gained an interest from the research society due to the ability of *Emiliania huxleyi, Prymnesium parvum* and *Phaeocystis pouchetii* to produce dimethylsulfide (DMS), a compound important for the cloud formation.

3.4. Raphidophyceae

Raphiodphyceans are registered in both marine and freshwater systems, and their fish-killing potential is known from mariculture in Japan, New Zealand, Chile and Canada (Hallegraeff and Hara 2004), with *Heterosigma akashiwo*, *Chattonella* spp., and *Fibrocapsa japonica* reported as causative species. Recently a new Raphidophyceae, *Viridilobus marinus*, described by Demir-Hilton et al. 2012 was co-occurring with *Heterosigma* in waters off USA. Raphidophyceans are susceptible toward common fixatives as formaldehyd and Lugol's solution and the presence of these algae in preserved material are easily overlooked; hence their occurrence and geographical distribution may be unreported. Several toxic mechanisms have been suggested: reactive oxygen species (ROS), brevetoxin-like compounds, haemagglutination and haemolytic activity, as well as mucus excretion clogging the gills. Even if the ichthyotoxicity of raphidophytes still is a hot topic among researchers (see for example Dorantes-Aranda et al. 2013; Kwak et al. 2012; Boer et al. 2011; Pezzolesi et al. 2010; Boer et al 2009); the research community has yet to conclude on the fish-killing properties of Raphidophyceans.

Fish mortality and economical loss due to *Heterosigma* are known from a wide range of countries with aquaculture industry; some examples are the recurrent blooms in Japan in 1972-1987 and the bloom in Canada in 1997 extending over a period of four months (Smayda 2006). The first reported mass occurrence of *Heterosigma* in European waters is the bloom in the Oslofjord, Norway, in 1964. Later on several blooms of *Heterosigma* with subsequent mortality in fish farms have been recorded from Spain, Norway, Ireland, and Scotland, as well as a report on bivalve mortality from Portugal (Smayda 2006). Furthermore, there have been discussions on wherever *Heterosigma* is an introduced or indigenous species in European waters. Based on available research Smayda (2006) concludes that *Heterosigma* belongs to the latter. A special feature of *Heterosigma* is that it has a very wide niche; hence blooms have been recorded during different nutritional conditions, varying stratification, in areas with freshwater influence or high salinity, as well as in pristine areas and in aquaculture-rich areas (see Smayda 2006 for further elucidation).

Recent phylogentic studies of *Chattonella* spp. have led to taxonomic revisions of this genera with *C. verruculosa* being transferred to the Dictyocophyceae (Hosoi-Tanabe et al. 2007; Edvardsen et al. 2007; Eikrem et al. 2009; Chang et al 2012); and evidence that *C. ovata* and *C. antiqua* are varieties of *C. marina* (Demura et al. 2007). According to these findings *Chattonella* at present consists of three species; i.e. *C. subsalsa, C. minima,* and *C. marina* (with three varieties *marina, antiqua* and *ovata*). The first recorded HAB of *Chattonella* with fish mortality is from India; thereafter *Chattonella* has become infamous for massive killings of farmed fish in Indian, Japanese, Korean, and Australian waters, as well as in the waters off USA (Imai and Yamaguchi 2012). Moreover, Imai and Yamaguchi refer to episodes where *Chattonella* has damaged local fisheries. Zingone et al. 2006 reports that *C. subsalsa* has been recorded in the Tyrrhenian Sea, but to their knowledge there have been no harmful events in that area.

Blooms of *Fibrocapsa* are known from Japan with subsequent fish mortalities. The first evidences of *Fibrocapsa* in European waters came from France (Billard 1992), the Netherlands (Vreling et al. 1995) and Germany (Elbrächter 1999). Later on *Fibrocapsa* is also reported from the Adriatic Sea and Tyrrhenian Sea (see Cucchiari et al. 2010). So far there are no reports on *Fibrocapsa* and fish-kills in

European waters. Boer et al (2009) have, however, studied the possibility of future harmful events on fish in Dutch waters, and they conclude that the risk will increase with climate change. From the Adriatic Sea, however, discolouration from *Fibrocapsa* blooms is reported to be nuisance towards tourism and recreational activities (Cucchiari et al. 2010).

3.5. Dictyochophyceae

In 1984 salmon and trout farmed in Danish waters died off during a bloom of the naked form of the silicoflagellate *Dictyocha speculum* (syn. *Distephanus speculum*); being the first report of *D. speculum* as a HAB species (see Smayda 2006 and references therein). Smayda also refers to mortality in mariculture in connection with blooms of siliceous *D. speculum* in Spain (salmon, turbot, and octopus), France (salmon) and Shetland (salmon). Moreover, he discuss wherever the bloom of the unidentified Flagellate X reported to be the cause of fish kills in Scottish lochs in 1979 and 1982 was a mixture of three known ichthyotoxic flagellates; the naked *D. speculum, Heterosigma akashiwo* and *Chattonella* cf. *verruculosa.*

The mechanism behind the fish-kills is not known, but both the siliceous and the naked form are thought to irritate the gills causing mucus production, oedema and hyperplasia, as well as hypoxia and hypercapnia. Smayda also refers to a HAB event in the Adriatic Sea with benthic mortality as a consequence of the decomposition of an extensive bloom of *D. speculum* creating anoxic conditions in the bottom waters. *D. speculum* has a complicated life cycle alternating between two motile stages, including a siliceous and a naked form, and a non-motile multinucleate stage. Smayda reports that the siliceous stage is more common in open, coastal waters, while the naked form tends to dominate in more enclosed, onshore areas. Interestingly, he has reviewed several papers on silicoflagellate blooms in European waters and the findings are all pointing in the same direction with mass occurrences developing in connection with nutrient enrichment of either terrestrial or anthropogenic origin. These studies have all demonstrated that nitrogen is the most important prerequisite for bloom development. Another interesting feature is that the alternating between the two stages makes *D. speculum* less

dependent on silica than diatoms, hence *D. speculum* can bloom after the diatoms have become Si-depleted.

The first reported bloom of *Pseudochattonella* (Hosoi-Tanabe et al. 2007; Edvardsen et al. 2007; Eikrem et al. 2009), previously identified as the raphiodphyte *Chattonella* aff. *verruculosa,* in European waters was in April-May in 1998 (Naustvoll 2006). Naustvoll, however, reports that reanalysis of material from the Swedish west coast have shown that *Pseudochattonella*-like cells have been present in low concentrations in this area since 1993. Pettersson and Pozdnyakov (2013) have recently published an analysis of the blooms of *Pseudochattonella* in Skagerrak, Kattegat and the German bight during the last two decades, giving an overview of the blooming events from 1998 to 2009. In 1998 the bloom originated off the Danish west coast in April; and spread northwards along the Danish coast to Kattegat and Skagerrak, most of the bloom is, however, thought to have been advected directly from Denmark to southern parts of Norway. In May Norwegian salmon-farmers located on the Skagerrak coast reported on increased fish mortality, and high concentrations of *Pseudochattonella* was found in samples collected in the proximity of the farming sites. Furthermore, there were reports on mortality of wild fish in Danish waters, including herring, mackerel and eels. Also in 2001 *Pseudochattonella* was blooming in Skagerrak/Kattegat with subsequent mortalities of farmed salmon in Norwegian coastal waters. During the last years the recurrent blooms of *Pseudochattonella* have become a problem for the Danish aquaculture industry. As a consequence a monitoring program on the presence of *Pseudochattonella* (and other HAB species) in Danish waters has been established for risk assessment (Andersen 2007).

Pseudochattonella in European waters is thought to be an invasive species which is now established with recurrent blooms in both Skagerrak and Kattegat (Naustvoll 2006). The work performed by Riisberg and Edvardsen (2009) have demonstrated that there were two distinct species during the bloom in 2000 with *P. farcimen* in Scandinavian waters and *P. verruculosa* in the German bight. Moreover, *P. farcimen* have so far only been identified in samples from Scandinavia, and Riisberg and Edvardsen indicates that *P. farcimen* might be indigenous; while their research supports that the European *P. verruculosa* is an introduced species. However, they stress the need for further research

on *Pseudochattonella* from different geographic regions before concluding on wherever *P. farcimen* is an indigenous species in Skagerrak and Kattegat or not.

There are still uncertainties on the ichthyotoxic principles that *Pseudochattonella* have on fish. A study by the Norwegian School of Veterinary Medicine on caged salmon exposed to *P. farcimen* during the bloom in 2001 set gill necrosis and bleeding in the orbital cavity in connection with the exposure to *Pseudochattonella* (referred by Skjelbred et al 2011). Skjelbred et al (2011) investigated the ichthyotoxic mechanisms of *Pseudochattonella* spp. and concluded that there are indications on metabolites affecting fish cell lines and gills of cod and salmon larvae. Moreover, Dittami et al (2012) have recently identified metabolic pathways that might be involved in the production of toxic compounds in *P. farcimen.*

Recently *Chattonella globosa* (Raphidophyceae) was transferred to Dictyochophyceae and renamed *Vicicitus globosus* (Chang et al 2012). Interestingly, Lømsland et al (2010) reported on the co-occurrence of *C. globosa, Dictyocha fibula* and algal cells having features indicating intermediate stages between these two species in connection with mortality in salmon-farms located in southern Norway in 2008. Furthermore, they suggest that *C. globosa* might be the naked form of *D. fibula.* Unfortunately their observations were only documented by light microscopy. Imai and Yamaguchi (2012), however, refer to a Japanese study which seems to be the first to document the phylogenetic connection between *C. globosa* and *D. fibula.* Furthermore, according to the review on *Chattonella* by Imai and Yamaguchi (2012), these researchers suggested a transfer of *C. globosa* to the Dictyochophycea.

3.6. Cyanobacteria

There are freshwater, brackish and marine species among the cyanobacteria and the prevalence of cyanobacterial blooms are generally larger in freshwater bodies than in brackish and marine waters. Several cyanobacteria are toxin producers, and the toxins can be grouped according to their target organ in mammals, i.e. hepatotoxins (microcystins, nodularins and cylindrospermopsis), neurotoxins (anatoxins, saxitoxins, palytoxins and beta-methylamino-L-alanine [BMAA]), skin (aplysiatoxins,

lyngbyatoxin), gastro-intestinal tract (lyngbyatoxin), and irritants (lipopolysaccharides); O`Neil et al 2012. In the Baltic Sea and adjacent waters (i.e. Gulf of Finland and the Bothnia Sea) blooms of cyanobacteria are a common summer phenomenon; and the bordering countries Denmark, Sweden and Finland have all established regular monitoring programs to follow the development and progress of cyanobacterial HAB events. The primary bloom-forming genera are the non-toxic *Aphanizomenon* and the toxin-producers *Nodularia* and *Anabena*; with *N. spumigena* being the dominating specie. There are several reports on animal intoxications from the Baltic Sea, and in a study on the possibility of fish acting as a vector for toxin transfer through the food web Sipiä et al 2007 found indications that three-spined stickleback (*Gasterosteus aculeatus*) could act as a vector.

Strictly marine cyanobacteria are found among the genera *Lyngbya*, *Trichodesmium* and *Synechococcus.* However, blooms of marine cyanobacterial species are only reported from tropical and sub-tropical waters. Generally *Lyngbya* spp. are benthic mat forming species, while species belonging to *Tricodesmium* and *Synechococcus* are pelagic. *L. majuscula* is reported to form HABs; with the first incident in Hawaii in the 1950s, and more recent in waters off Australia, Florida and the Caribbean (O´Neil et al. 2011). As reviewed by O´Neil and colleagues (2011) humans exposed to HABs of *L. majuscula* report on dermatitis and asthma-like symptoms. Furthermore, *L. majuscula* blooms may have severe consequences; Yasumoto (1998) demonstrated that consumption of poisonous green sea turtle meat (lyngbyatoxin A) was the cause of a fatal intoxication in Madagascar. As summarized by O´Neil et al (2011) *Synechococcus* is cosmopolitan, with long-lasting blooms that can cover large areas. They report that *Synechococcous* blooms may have a negative impact on the ecosystem as a whole; anoxic conditions, increased light attenuation impacting benthic communities negatively, and inhibition of zooplankton grazing are reported. Previously only freshwater species of *Synechococcus* were known to be toxin producers, but Carmicheal and Li (2006) demonstrated that a haline strain of *Synechococcus* is able to produce microcystin.

Trichodesmium may form large blooms in oligotrophic waters; with reports on blooms from the Pacific, Arabian Sean, Indian Ocean, Gulf of Mexico and the Caribbean (O´Neil at al 2011). An Australian study (Negri et al. 2004) implicates that the observed mortality among farmed pearl oysters

was caused by malnutrition during a large *Trichodesmium* bloom, and not as first suspected toxins produced by the cyanobacteria. There are also reports that anoxic conditions due decaying *Trichodesmium* have resulted in mortality among marine organisms (i.e. fish, shrimp and oysters); see Negri et al 2004 and references therein. There are reports on human reactions after exposure to *Trichodesmium* with headaches, skin-irritations and asthma-like symptoms. Moreover, ciguatera-like intoxications have been reported from Australia after consumption of mackerel; and recently palytoxin and a derivate have been isolated from both *T. erythraeum* and *T. thiebautii* (O´Neil et al 2011). This finding implicate that not only dinoflagellates, but also cyanobacteria might be involved in CFP. O´Neil et al (2011) also reports that there are indications that *Trichodesmium* produce other toxins; i.e. microcystins, cylindrospermopsin and saxitoxin.

4. Monitoring in Europe

In Europe, monitoring of algal toxins in shellfish is regulated by EC directives and guidelines. Regulations set maximum levels for Paralytic Shellfish Poisoning (PSP) toxins (e.g. saxitoxin), Diarrhetic Shellfish Poisoning (DSP) lipophilic toxins (including the okadaic acid group and the pectenotoxin group), Amnesic Shellfish Poisoning (ASP) toxins (e.g. domoic acid) as well as two other groups of lipophilic marine toxins: the azaspiracid group (AZA) and the yessotoxin group (YTX). Regulation 854/2004/EC mandates the competent authorities in the EU Member States to examine live bivalve molluscs for the presence of marine biotoxins before being placed on the market. With regard to methodologies, Annex III of EC Regulation No. 2074/2005/EC established recognised testing methods for marine biotoxins, including biological methods (mouse bioassay and rat bioassay) in the case of PSP and lipophilic toxins, as reference methods and chemical methods (for ASP toxins). This has recently been amended by EC Regulation No. 15/2011. This new regulation establishes that the reference method for the detection of lipophilic toxins should be a validated technique of liquid chromatography and mass spectrometry, available for consultation on European Union Reference Laboratory, EU-RL. The EU-RL ensures a standardized system throughout the EU by coordinating the European Network of National Reference Laboratories. In addition to monitoring of biotoxins most European countries also have a regular monitoring program for the detection of the corresponding

source organisms. It has been shown that the toxin content of source species depend on several factors and among them geographical differences have been identified (Tillman et al 2009, Montoya et al 2010). Therefore threshold limitis for the toxin producing microalgae may differ between European countries.

5. Future Trends

From a global perspective, a growing population puts the need for a stable supply of healthy and nutritious food on the agenda. FAO (2012) has, in this context, stated that the fisheries and aquaculture sector offers opportunities to increase food and nutrition security, alleviate poverty, generate economic growth and ensure improved use of resources. Stimulated by the higher demand for seafood they have projected that the world fisheries and aquaculture production will reach about 172 million tons in 2021, with most of the growth in the aquaculture sector.

There is an increasing concern regarding climate change and the effect on phytoplankton, and HAB species especially and their implications for human health (Balbus et al. 2013, Hallegraeff 2010, Moore et al. 2008, Hays et al. 2005). As summarized by Hallegraeff (2010) the following effects from climate change on HABs may be expected: 1) range expansion of warm-water species at the expense of cold-water species, which are driven poleward; 2) species-specific changes in the abundance and seasonal window of growth of HAB taxa; 3) earlier timing of peak production of some phytoplankton; and 4) secondary effects for marine food webs, notably when individual zooplankton and fish grazers are differentially impacted ("match-mismatch"). Furthermore Hinder et al. (2011) have performed an analysis of the effects climate change may have on HABs and shellfish safety in UK, and they concluded that a potential future climate change could bring new toxic species into the surrounding UK waters. In this context they stress the necessity of having a well-functioning monitoring program to ensure the detection of emerging HAB species and subsequent accumulation of emerging toxins in shellfish. Thus changes in the climate may lead to changes in the species composition and introduction of new and potentially toxic species that the present detection systems do not reveal.

Furthermore, there is a political consensus in Europe on the need to reduce experiments on animals and the European Commission has stated that this can be done by the introduction of alternate methods that can replace animal testing[1]. Norway and Ireland have throughout the years played an active role to reduce the present use of mouse bioassays (MBA) to detect algal toxins in shell meat, and from 2015 chemical analyses will be the only permitted regulatory method in the European community. MBA will then only be used as a screening tool for novel or emerging toxins in European waters. MBA, however, only give information on the presence of toxins and are not suitable for identification of the toxin itself, and scientists have therefore suggested looking into "omics" as a new tool for screening and detection of biotoxins (Wu & Richardson 2011, Fleming et al. 2006). At present, most of the effort has been within proteomics (reviewed by e.g. Campos et al. 2012, Giacometti et al. 2012, Piñeiro et al. 2010). Mondeguer et al. (2012) proposed metabolomics as a new, non-targeted method for the detection of algal toxins in shellfish. Moreover they emphasized the need for research on indirect and, if possible, generic biomarkers.

The oceans are globally an important food provider and by means of an increasing population the need for healthy and safe seafood will be even more important (FAO 2012). The Intergovernmental Panel on Climate Change (IPCC)

Further research and knowledge on marine biotoxins, their producers and under which oceanographic conditions they become harmful will be essential, especially will near real-time monitoring systems play an important role in seafood safety.

[1] http://ec.europa.eu/environment/chemicals/lab_animals/alternative_en.html

6. Web Resources

IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae;

http://www.marinespecies.org/HAB

AlgaeBase; http://www.algaebase.org

Nordic microalgae and aquatic protozoa; http://nordicmicroalgae.org/

The European Union Reference Laboratorty for Marine Biotoxins;

http://www.aesan.msssi.gob.es/en/CRLMB/web/home.shtml

Information on the algal situation in Norwegian coastal waters; http://algeinfo.imr.no/

Global Ecology and Oceanography of Harmful Algal Blooms; http://www.geohab.info/

The Intergovernmental Oceanographic Commission of UNESCO Harmful Algal Bloom Program;

http://ioc-unesco.org/hab/

Information on the algal situation in Irish coastal waters;

http://www.marine.ie/home/publicationsdata/data/Habs+Search+Database/PhytoplanktonShellfishToxicitySummary.html

Information on shellfish safety in Norway; http://www.matportalen.no/verktoy/blaskjellvarsel/

Information on the algal situation in the Baltic Sea;

http://www.smhi.se/en/Weather/Sweden-weather/the-algae-situation-1.11631;

http://www.dmi.dk/en/hav/satellitmaalinger/algal-maps/

http://www.itameriportaali.fi/en/tietoa/algaline_seuranta/en_GB/algaline_seuranta/

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Table 1 Diatoms producing domoic acid from Moestrup et al. 2013.

Current name		Environment	Distribution
Halamphora coffeaeformis	(Agardh) Levkov 2009	Brackish	Type localities Karlovy Vary, Cardigan Bay
Nitzschia navis-varingica	Lundholm and Moestrup 2000	Marine	East Asia and Australia, tropical to subtropical
Pseudo-nitzschia australis	Frenguelli 1939	Marine	Temperate and subtropical
Ps. calliantha	Lundholm, Moestrup and Hasle 2003	Marine	Cosmopolitan (?)
Ps. cuspidata	(Hasle) Hasle 1993	Marine	Temperate and warmer waters
Ps. delicatissima	(Cleve) Heiden 1928	Marine	Arctic, temperate and subtropical
Ps. fraudulenta	(Cleve) Hasle 1993	Marine	Temperate and subtropical
Ps. galaxiae	Lundholm and Moestrup 2002	Marine	Subtropical (?)
Ps. multiseries	(Hasle) Hasle 1995	Marine	Temperate and subtropical
Ps. multistriata	(Takano) Takano 1995	Marine	Subtropical
Ps. pseudodelicatissima	(Hasle) Hasle 1993	Marine	Temperate (?)
Ps. pungens	(Grunow *ex* Cleve) Hasle 1993	Marine	Temperate, subtropical and tropical
Ps. seriata	(Cleve) Peragallo 1899	Marine	Cold water species
Ps. turgidula	(Husted) Hasle 1993	Marine	Arctic (?), temperate and subtropical

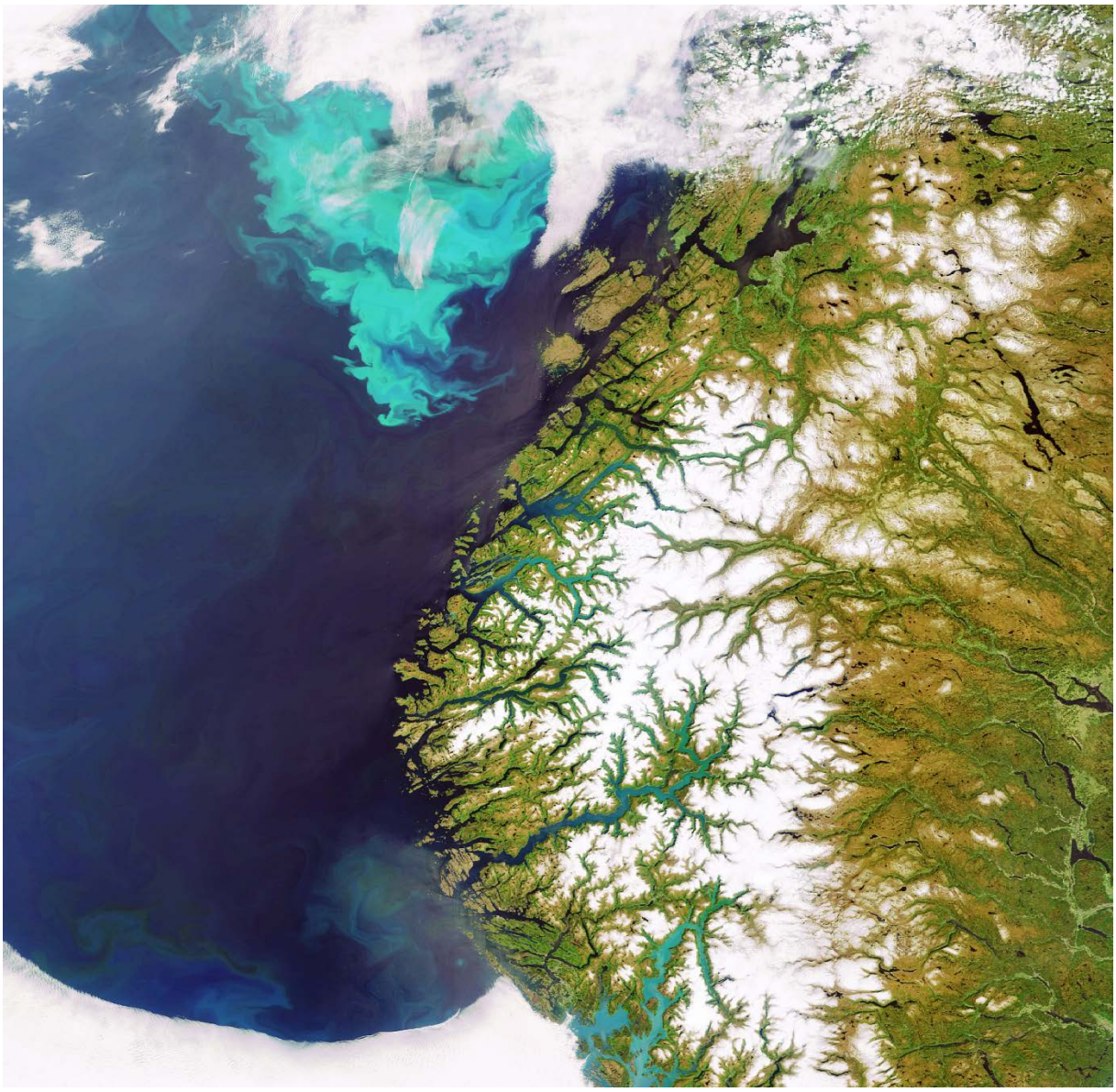

Figure 1 ***Emiliania huxleyi*** **bloom off the Norwegian coast in 2006. Photo: ESA.**

Chapter 7

Biofuels from Microalgae

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Microfluidics and *in situ* Sensors for Microalgae

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1. Abstract

Phytoplankton are important in global biogeochemistry since they produce the bulk of oxygen on Earth through photosynthesis. They form the base of the marine food and are primary producers of organic carbon. Some species produce polyether toxins, thus forming harmful algal blooms that can have detrimental effects on local fauna and flora. For these reasons, phytoplankton monitoring in the oceans is critical. It can provide insight to climate change, microbial ecology, biogeochemical cycles and health toxicity of recreational waters. Currently, the oceans are vastly under-sampled and *in situ* sensors and sensor networks can address this need for sampling. Miniaturised and microfluidic sensors are increasingly being used for phytoplankton monitoring with great promise for future applications. This chapter will provide an overview of traditional methods of phytoplankton analysis, introduce microfluidics, and review the state-of-the art in miniaturised and *in situ* sensors for algal detection in the oceans.

2. Introduction

Data collection in the oceans is typically done during organised cruises. These expeditions can be very expensive, labour-intensive and usually target pre-defined locations. As a consequence, the oceans are vastly under-sampled; only about 5 percent of the 1.3 billion cubic kilometres covering Earth have been explored until now (Schofield et al., 2013). Samples are collected in the field and then analysed on-board research vessels or preserved for *ex situ* studies in laboratories. However, not all phytoplankton can be preserved, which results in preservation bias.

Due to the massive volume of water that would need to be sampled for complete characterisation of global marine microbial taxonomic and functional diversity, and of microalgae in particular, the task is almost insurmountable using solely data/sample collection from research cruises. Remote sensing using ocean colour analysis of satellite images is a more cost-effective approach to obtain information on phytoplankton biomass, based on chlorophyll concentration (Blondeau-Patissier et al., 2014). Its disadvantages are that data cannot be obtained in cloudy weather, are limited to first optical depth and spatial resolution can be low.

Autonomous Underwater Vehicles (AUVs) and other complementary platforms implement data collection for phytoplankton in the oceans by housing *in situ* and deployable sensors (Schofield et al., 2013). A sensor is defined as an integrated analytical device that does not require reagent addition and includes a recognition element for analyte detection, as known as a transducer (figure 1). This can be a substance or a device that converts the sensing signal to a detectable output, quantitative or qualitative, such as electrical energy or light. A biosensor, in particular, has a biological transducer, which would be an enzyme, antibody or whole cells.

Figure 1: A simple diagram of a sensor. The sample is prepared for analysis and the transducer senses a signal input. The signal is converted to a detectable ouput and then interpreted.

Sensors for phytoplankton can be deployed while completely submerged, while their components include sample processing, analysis and data storage. The majority of these platforms are not simply a sensor, as described by the definition, but large integrated systems with multiple sensing components. They can provide biological, physical, chemical or optical information by employing analytical tools, that are portable versions of the equipment used *ex situ* in the lab or on-board research ships for phytoplankton analysis.

The state-of-the-art for lab-on-chip technologies for algal detection using optical and impedance methods (Schaap et al., 2012a), *in situ* sensors for phytoplankton (Erickson et al., 2011) and nutrient (Mills and Fones, 2012) analyses have been reviewed in 2012. In this chapter, we will review the most significant work in the prior art using all detection methods (biological, physical, chemical, including optical) as well as focus on advances in the field after 2012.

3. Traditional Methods for Analysis of Microalgae

The most common methods of algal analysis and identification are optical and electron microscopy (Tomas, 1997). Cells are stained with fluorescence or immunological methods to assist visualisation. This process is time-consuming and requires specialised cytologists. Alternatively, computer-based microscopy image recognition analyses can be used to identify and count phytoplankton (Embleton et al., 2003) with open software, PlanktoVision, recently developed (Schulze et al., 2013). Light scattering, particle sizing and spectrophotometry are also used for initial size and population information.

Photosynthetic pigment identification can be used to distinguish between different phytoplankton species (Jeffrey et al., 2011). Non-fluorescent algal pigments are detected with High-Pressure Liquid Chromatography (HPLC) (Bidigare et al., 2005; Irigoien et al., 2004) and Liquid Chromatography Mass Spectrometry (LC-MS) (Airs and Garrido, 2011). Recent papers have used micro-Raman spectroscopy to map carotenoids in *Chlorella sorokiniana* and *Neochloris oleobundans* (Huang et al., 2010; Pilát et al., 2012).

Fluorometry is used for bulk characterisation of phytoplankton fluorescence. Flow cytometric analysis of phytoplankton yields quantitative data based on the auto-fluorescence of cells from their photosynthetic pigments, but also their size and light scattering properties (Sosik et al., 2010). It has been used as a shipboard tool since the 1980s (Olson et al., 1985). In particular, Fluorescence-Activated Cell Sorting (FACS) can separate a heterogeneous population of algae in taxonomic classes with respect to their fluorescent pigments. Some examples of pigments are: chlorophyll (found in all phytoplankton) emits red fluorescence when excited at 488 nm; phycoerythrine (more abundant in cyanobacteria/cryptophytes) emits orange fluorescence when excited at 488 nm; and phycocyanine (present in cyanobacteria/cryptophytes) emits red fluorescence when excited at 633 nm.

Native algal cells from the environmental samples obtained in the field can be isolated and either preserved for future analyses or grown in the lab using microbiological methods. This allows for further investigations and has resulted to several culture collections available word-wide. In the

United Kingdom, these are the Plymouth Culture Collection of Marine Microalgae (CCMM) in England, and the Culture Collection of Algae and Protozoa (CCAP) in Scotland. The US-based national collection is housed at the National Center for Marine Algae and Microbiota (CMAM), formerly known as the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP).

Cells can then be further analysed using molecular biology methods (Zehr et al., 2009). Nucleic acids found in minute amounts in cells require amplification to detectable limits for further analyses by nucleic acid amplification technologies (NAAT). DNA can be amplified by polymerase chain reaction (PCR) (Mullis et al., 1987) and RNA with reverse transcriptase-PCR (RT-PCR). More recent amplification alternatives, termed as isothermal, operate at lower temperatures than PCR and do not require thermo-cycling; their state-of-the-art has been recently reviewed (Yan et al., 2014). However, amplification of genes of interest by NAAT creates bias when studying microbial diversity. Molecular methods without DNA amplification for the investigation of mixed community phytoplankton samples include genome sequencing, termed as genomics, and metagenomics (Heidelberg and Heidelberg, 2005; Rynearson and Palenik, 2011). Metagenomics or community genomics, unlike genomics, do not rely on isolating individual species and applies Sanger or pyro-sequencing methods to environmental samples of entire communities. The United States Joint Genome Institute sponsored by the Department of Energy collects and catalogues algal genomic data. A special mention has to be made on J. Craig Venter's approach for building a metagenomic profile of the oceans during the global ocean sampling expedition on board the Sorcerer II sailing vessel (Rusch et al., 2007; Williamson et al., 2012). Phytoplankton proteomics (Jones et al., 2011) and metaproteomics (Siggins et al., 2012) are far less explored areas, as is lipidomics (Li et al., 2014) with only a handful of studies as of yet.

Some mainstream bench-top molecular tools have been further optimised specifically for microbial community analysis applications. Firstly, the PhyloChip is a microarray with 297,851 immobilized probes for 16S rRNA genes (DeSantis et al., 2007) on a glass chip, developed by the Lawrence Berkeley National Laboratory (USA). The 25-mer probes are complementary to 842 bacterial and

archeal subfamilies and can provide with high precision community information from a single sample. The principle of operation is that DNA or DNA extracted from an environmental sample is first labelled with a fluorescent dye. The native fluorescent DNA is then allowed to hybridise with the probes and any unbound nucleic acids are washed off. The signal intensity corresponding to different probes is finally quantified. Secondly, the GeoChip also follows the microarray design concept with 167,044 50-mer oligos covering 395,894 coding sequences from bacteria, achaea, fungi and viruses (He et al., 0000). Thirdly, the Biotrove Open Array system has been used extensively for environmental samples (van Doorn et al., 2009). It allows high throughput qPCR reactions in 33 nL volumes. A total of 3072 reactions can be run in each plate and real-time fluorescence is monitored using SYBR or Taqman probes.

4. Microfluidics or lab-on-chip

Microfluidics is the science and technology of systems that process or manipulate small amounts of fluids using channels with dimensions from one to 1000 micrometres (Whitesides, 2006). The field is largely interdisciplinary, combining aspects of engineering, physics, chemistry and biology. The concept of miniaturised total chemical analysis systems was presented in the early 90s by Andreas Manz (Manz et al., 1990), although some embryonic elements were introduced in the 1950s and later developed to the current inkjet printing technology. The early work of Manz *et al* on a miniaturised high-pressure liquid chromatography (HPLC) column microfluidic device (Manz et al., 1990) initiated the field that is now widely known as "lab-on-chip". Since then a myriad of devices has been developed for applications varying from environmental analysis (Li and Lin, 2009), forensics (Horsman et al., 2007) and complex clinical samples (Crevillén et al., 2007).

A microfluidic platform can perform the same set of operations as larger fluidic alternatives for chemical and biochemical processes and reactions typically taking place in specialised laboratories (Mark et al., 2010). The advantages of microfluidic systems are; (i) ease of operation without the need of highly trained personnel; (ii) reduced reagent consumption, cost of manufacture and waste generation; (iii) low power usage; (iv) and significantly smaller size. Lab-on-chip devices also allow parallelisation of reactions and thus yielding faster results with better statistics. Systems can be modular and mimic *in vivo* conditions. Finally, single cell and molecule applications are feasible with microfluidics since they match well with the small size of systems.

Devices can be fabricated in polymer substrates (thermoplastics or elastomers) or in glass, both having the advantage of being optically clear. A large variety of approaches to particle separation, fluid manipulation, device fabrication and operation have been presented. A few examples will be highlighted hereon. Continuous flow is typically affected using pumps, mixers and valves. One example is the Quake pneumatic valve constructed from polydimethylsiloxane (PDMS) by soft-lithographic procedures (Hansen et al., 2002). It operates by restriction of a microfluidic channel by pressure and allows design of complicated chips with multiple fluidic paths. Alternative ways of fluid

manipulation other than continuous flow are droplet microfluidics or digital microfluidics. Droplet microfluidics uses discreet mono-disperse droplets inside microfluidic channels formed by two immiscible fluids like oil and water (Teh et al., 2008). Digital microfluidics or electrowetting-on-dielectrics (EWOD) operates by applying a potential to an electrode coated with a hydrophobic insulator (Zeng and Wang, 2013). This modifies the contact angle of a drop of liquid lying on top of the electrode and allows mixing, dispensing and merging of very small droplets of water in oil (Choi et al., 2012). Dielectrophoresis subjects particles to an AC field in order to induce a temporary dipole moment (Morgan et al., 1999). This allows accurate particle separation based on their polarisability.

Applications of microfluidics range from biological and chemical assays (Fair et al., 2007), chemical sensing (Wu and Gu, 2011) to optics (Tang et al., 2008), drug delivery (Khan et al., 2013), cell (Huang et al., 2002) and molecular (Kim et al., 2007) separation. As highlighted by George Whitesides in 2006 (Whitesides, 2006), advances in molecular analysis, biodefense, molecular biology and microelectronics are contributing to the field. The global market growth for microfluidic devices has been estimated to $5.7 billion by 2018 (Roussel, 2013), mostly driven by pharmaceutical companies for healthcare diagnostics.

There are two main examples of lab-on-chip devices that have been commercialised and are used for mainstream applications. Firstly, the Agilent Bioanalyzer is a microfluidic system, based on capillary electrophoresis, for analysis of nucleic acids and proteins. Secondly, the BioMark HD System from Fluidigm incorporates 9216 qPCR reactions on one single chip.

There is still large scope for innovative commercial devices since a complete sample-to-answer integrated system has yet to be developed. Many individual microfluidic components have been presented but major challenges still lie with sample preparation (Labuz and Takayama, 2014; Mariella, 2008) and system integration (Nge et al., 2013). Nevertheless, advances in the lab-on-chip field by 2014 have made the possibility of microfluidic devices becoming widely-used products a not too distant reality (Whitesides, 2014).

5. Deployable and *in situ* Sensors for Microalgae

5.1. Platforms and Sensor Networks

There is a large range of complementary platforms in addition to research ships and satellites for sampling and data acquisition on phytoplankton and microbial ecology (Shade et al., 2009). Stationary, fixed-point or Eulerian platforms are moorings, buoys and cabled observatories that offer high temporal resolution (Lampitt et al., 2010).They can offer large power capabilities and limited restriction on the size and shape of sensors that they can accommodate. However, they need to operate in large networks to provide spatial resolution (Adornato et al., 2010). Conversely, AUVs, gliders and Argo floats are unmanned mobile devices that operate autonomously, each type to a different extent. They can carry a variety of sensors and be deployed for up to months at a time (Schofield et al., 2013). Their drawbacks are significant limitations on power and size of payload. Both stationary and mobile platforms suffer from biofouling due to long-term exposure to the marine environment (Delauney et al., 2010).

5.2. Sensors for Morphology Observations

As *in situ* alternatives to microscopy in a lab, the majority of sensors employ light scattering, spectrophotometry, fluorometry and imaging methods to analyse the size and shape of microalgae. Microfluidic systems for phytoplankton identification have been presented for impedance spectroscopy and optofluidic monolithic devices. These microsystems have not as of yet been deployed in the field, but show potential to integration to AUVs because of their small size and power consumption. An excellent review on optical tools available for ocean monitoring was published in 2009 (Moore et al., 2009).

5.2.1. **Light Scattering**

Light scattering sensors are often referred to as turbidity sensors. They provide with information of particle size, composition and distribution of populations. They operate on the principle that light scatters in all directions when it hits a particle. Forward scattering correlates to the particle size and

side scattering is indicative of particle shape. A large range of commercial turbidity sensors have available (Moore et al., 2009) and established configuration standards like ISO 7027 exist.

The Bellouard group in the Netherlands developed an optofluidic microdevice for algae identification (Schaap et al., 2012a). A glass device, comprising of a straight channel and a curved waveguide, was etched with regions of higher refractive index using a femtosecond laser (Davis et al., 1996). A 1550 nm laser excited algae passing through the microfluidic channel, producing fluctuations in optical intensity measured by a photodetector. Images of the individual algae were also captured by a video camera implemented on the device. The acquired wave-functions identified up to five separate classes of algae in a spiked sample with an accuracy of 78% (Schaap et al., 2012b). Environmental samples would be harder to discern on this system due to detritus present and dilutions needed until only one cell was present in the detection zone at any one time. However, the monolithic nature of the device shows great promise for future adaption to a deployable sensor.

5.2.1. **Spectrophotometry**

Spectrophotometry measures the light absorbed by a sample as function of wavelength after illumination with broadband radiation or white light. An *in situ* spectrophotometer, the "*Brevebuster*", is available commercially for identification of *Karenia brevis* blooms (Robbins et al., 2006). The sensor uses a tungsten/deuterium light for illumination and a fiber optic spectrophotometer for detection. It has been used on moorings, buoys and ships (Kirkpatrick et al., 2011). The National Oceanic and Atmospheric Administration (NOAA) deploys a network of these "*Brevebuster*" systems on fixed platforms in Tampa Bay (Florida, USA) for automated detection of red tides

5.2.2. **Fluorometry and Bioluminescence**

Fluorescence is the process of light emission by a sample when excited by a specific stimulus. It provides with information that allows distinguishing algal cells from debris and other particles in seawater. Identification of major phytoplankton groups is also feasible based on the light absorbed by their predominant photosynthetic pigments and subsequently emitted as fluorescent light. A variety of commercial instruments are available and one indicative example for each type of fluorometers will

be illustrated in the following section. The list is by no means exhaustive and the author holds no commercial relationships with the mentioned companies.

Single channel fluorometers measure one single wavelength and are calibrated for detection of chlorophyll, phycocyanine or other pigments, and dissolved organic matter. The Amiscience handheld fluorometer by Beagle Bioproducts Inc (USA) is an example of a low-cost handheld instrument for single measurements *in situ* or in the lab. It can be calibrated for the detection of phycocyanine (600 nm excitation, 650 cm emission) and/or chlorophyll (440 nm excitation, 670 cm emission). The required sample volume is 200 µL with no pre-treatment necessary. The device has USB connectivity and a LCD touch-screen display user interface. The detection range is 10 to 100,000 ppb for phycocyanine and 0.25 to 2,500 ppb for chlorophyll.

Spectral fluorometers use either fluorescence emission or fluorescence excitation. The first method provides a spectrum of algal pigment fluorescence at a single excitation wavelength. The second method varies the fluorescence excitation wavelength and provides data on the absorption of different pigments. Wet Labs (USA) has developed the ECO range of fluorescence emission fluorometers. They operate at full ocean depth and provide with data on chlorophyll-a, coloured dissolved organic matter, fluorescein/rhodamin and phycorethrin/phycocyanine.

Excitation relaxation fluorometers measure changes in chlorophyll fluorescence on a very short timescale. One type of technology is Fast Repetition Rate Fluorometers (FRRF), which used by the FastOcean family of systems (Chelsea Technologies, UK). They allow calculations of the rate of absolute photosynthetic electron transport on functional photosystem II per unit volume, both in the lab and *in situ* (Oxborough et al., 2012).

Bioluminescence is the natural ability to produce and emit usually blue-green light at ~500 nm by dinoflagellate phytoplankton species, like *Alexandrium tamarense* (Haddock et al., 2010). A submersible sensor for use in moorings and towed vehicles is the GlowTracka (Chelsea Technologies, UK). A photodiode measures the light (as photons) produced by algae in the sample as a function of time. Seawater is flowed through the detector by a 28 mm pipe and the entire sensor weighs 2.4 kg.

Information on primary production of bioluminescent species is acquired and can be correlated to imaging data in order to identify the specific bioluminescent species.

5.2.3. **Imaging**

The Woods Hole Oceanographic Institute (WHOI) first developed the Video Plankton Recorder (VPR) (Davis et al., 1992). This is an underwater video microscope and has been deployed in AUVs, mooring and towed vehicles. The most recent version can image particles ranging from 100 µm to 1 cm at a field of view from 5 to 20 mm (Davis et al., 2005). The instrument is not commercially available but can be used via the Institute's pool of instrumentation.

The FlowCAM system was later developed at the Bigelow Laboratory for Ocean Sciences (USA) (Sieracki et al., 1998). The sample is drawn into a flow cell where digital images of particles are continuously acquired by a flash illumination from an LED. A CCD camera captures the image and image analysis software resolves the information. Fluorescence information is also recorded by a PMT, although the instrument does not operate in the same principle as a flow cytometer, not having sheath flow. FlowCAM is available commercially with its most recent VS series model specification offering imaging capabilities of particles in the size range of 2 µm to 2 mm at a flow rate of 3 mL/min. The system is portable with an integrated user interface comprising of a screen, keyboard and mouse in a hard case.

5.3. Flow Cytometry and Impedance Spectroscopy

Flow cytometry is used *in situ* by transferring bench-top instruments on board research vessels or by deployable versions of the bench-top instruments, usually tethered to a ship or to a stationary platform. A flow cytometer is defined as the instrument that measures and analyses signals from particles in a liquid stream passing though a beam of light (Givan, 2001). A sheath fluid is used to align and focus the particles so that they to flow in a single line. At any one time only one particle crosses the light beam, usually from a laser source; a detector then acquires an optical or electrical impedance signal. The latter is based on the principle of impedance spectroscopy, which measures the electrical properties of particles passing through a set of electrodes while applying an AC potential.

The first cytometric instrument developed in the Netherlands for phytoplankton study was the Optical Plankton Analyzer (OPA) (Dubelaar et al., 1989; Peeters et al., 1989). It detects a larger particle size range (<1 mm) than commercial flow cytometers (1-25 µm). Other design features specific to marine applications include a large dynamic range of 1000x factor and modular excitation/detection optics. A large flow cell and high flow rates are used to achieve these specific requirements. Cytobuoy is a commercially available floating standalone version of the original OPA system (Dubelaar and Gerritzen, 2000; Dubelaar et al., 1999) and can be deployed in a buoy. A portable bench-top CytoSense and submersible CytoSub have also been developed within the same family of instruments. They are very comparable to FlowCAM because they do not require an external sheath flow like typical flow cytometers. A wide and slow-shear flow cuvette is used to focus the sample. Seawater, which has been filtered through a capsule filter, is used as sheath flow liquid. Forward scatter, side scatter, orange and red fluorescence signals are detected by photodiodes and solid-state photomultiplier tubes. Excitation is by a single low power Nd: Yag laser.

At the same time, the FlowCytoBot was developed at WHOI (Olson et al., 2003). It is capable of light scattering and fluorescence particle analysis with an adjustable flow rate of 12.3 to 50 µL/min. It recycles sheath flow similarly to CytoBuoy and also uses a diode-pumped laser. An imaging FlowCytoBot was presented in 2007 (Olson and Sosik, 2007) and used to provide early warning for a *Dinophysis* bloom in the Gulf of Mexico in 2008 (Campbell et al., 2010). FlowCytoBot is optimised for smaller phytoplankton cells (1-15 µm) while its imaging version can visualise cells with larger diameters (10-300 µm). Both instruments have automated features for enumeration, identification and cell sizing and can be deployed for up to six months unattended. They include biofouling control and real-time fluidity sensing.

The main difference between the European OPA and American FlowCytoBot systems is their tethering capability. Cytobuoy uses batteries as a power supply and radio communications, whereas FlowCytoBot is a cabled instrument. Neither has been deployed on AUVs up to now. A commercial version of the Imaging FlowCytoBot is now licensed from McLane Research Labs.

Microfluidic flow cytometers could be deployed on AUVs, as their small size would fit the payload of the vehicles. Two lab-based micro-cytometers for phytoplankton have been published but neither has been tested on an autonomous vehicle yet. The first system to be developed was at the Morgan lab in Southampton (UK) for combined impedance spectroscopy and intrinsic fluorescence measurements (Benazzi et al., 2007). A PDMS chip was fabricated with a microchannel of 11-μm height and 20-μm width. Fluorescence was measured at three wavelengths with *Isochrysis galbana, Rhodosorus marinus* and *Synecococcus* tested as exemplar species. Results from this device with non-integrated optics compared well with those acquired from a bench-top flow cytometer. However, only one-dimensional flow focusing was used, in the width direction, and impedance measurements could not size the smaller species *Synecococcus*.

At the National Research Lab, the Ligler group developed a micro-cytometer for fluorescence only measurements with two-dimensional flow focusing (Hashemi et al., 2011a; Hashemi et al., 2011b). This was achieved using chevron-shaped grooves fabricated on the micro-channel walls. The chip has integrated optics for light scattering and up to three colours of fluorescence. The channel dimensions were at 390 μm by 130 μm and the chip was also fabricated from PDMS using standard micro-fabrication procedures. Phytoplankton species *Karenia brevis*, *Synechococcus* sp., *Pseudo-Nitzchia*, and *Alexandrium*, ranging from 1 to 50 μm in diameter, were analysed. The smaller in size, *Synechococcus*, was distinguished, which was not feasible with impedance measurements by the Southampton device. However, there was some overlap between the other populations of phytoplankton, making classification difficult.

Both microsystems have potential for deployment on AUVs if the large power requirements of the optics are improved. Another consideration is that the small size of the fluidic channels would be prone to clogging by debris and biofouling. The latter was addressed by the Southampton group, which tested different types of antifouling methods for engineering materials deployed in the marine environment (Meier et al., 2013). PDMS was a particularly bad candidate for deployable sensors, as it tended to swell in wet conditions. Chips from thermoplastics and glass had a better performance being more rigid materials.

5.4. Sensors for Speciation Using Molecular Tools

Sensors using molecular tools speciate and quantify phytoplankton by detecting nucleic acids, proteins, carbohydrates and lipids, that are characteristic for each species. Instruments in the field use: (i) direct binding of immunological (antibodies) or nucleic acid (rDNA) probes; and (ii) NAAT. Both approaches are limited to species that have already been isolated, cultured and partly or wholly sequenced. Probes can be electro-active, fluorescent or luminescent.

Three large deployable *in situ* systems have been developed for molecular detection of phytoplankton: the Environmental Sample Processor (ESP) at the Monterey Bay Aquarium Research Institute (MBARI, USA), the IISA-Gene in Japan and the Automated Microbial Genosensor (AMG) at the University of South Florida (USA).

The ESP is an autonomous and submersible system that collects, analyses and archives individual water samples in “pucks”, custom-designed chambers with pre-stored reagents (Scholin et al., 1998). A rotating carousel automates the process. It weighs ~27 kg and is a cylinder with a 56-cm diameter and 82-cm height. All the steps of sample processing occur under atmospheric pressure. Toxic diatom *Pseudo-nitzschia australis* was first detected on the ESP with an automated competitive ELISA immunoassay (Doucette et al., 2009). This species produces domoic acid, a tightly monitored amnesic shellfish poison. Samples were archived and stored on filters for fluorescence *in situ* hybridization (FISH) assays performed back in the laboratory (Greenfield et al., 2006). Fluorescent rRNA targeted DNA probes were used. The limit of detection achieved was 100 cells per litre. An additional module, the MicroFludics Block (MFB) was later developed for *in situ* solid-phase nucleic acid extraction and quantitative PCR (Preston et al., 2011). The DNA was extracted and purified from 100-1000 mL samples down to 50 µL eluent aliquots. Real-time PCR was performed from these samples and the results were broadcasted back to shore after amplification. This version of the system was deployed for 28 days in the Monterey Bay on a coastal mooring. Meta-transcriptomic analyses were also feasible from samples collected autonomously by the ESP and preserved in ambient temperatures until retrieval (Ottesen et al., 2011). RNA samples were filtered *in situ* and treated with preservative

RNALater. The ESP is now commercially available in the $250K price range by Spyglass Biosecurity Inc. Until now it has been deployed on AUVs, moorings, remotely operated vehicles and profiling drifters. Deep sea deployments of the ESP were also performed using both the qPCR and rRNA assays at 4000 m depth in the Santa Monica Basin (Ussler et al., 2013).

The IISA-Gene system uses microfluidic components that operate at ambient pressure for *in situ* PCR measurements (Fukuba et al., 2011). The sensor is very versatile because of the miniaturised components, and can operate at extreme depths. The only drawback is that it collects relatively small sample volumes of 0.5 mL per hour. All components of the analysis unit are immersed in fluorinated oil and are connected by underwater cables to a remote operator on a surface vessel. The most recent version of the instrument has 128 sampling channels for in-line parallel collection (Okamura et al., 2014).

The AMG is a deployable system which uses an isothermal NAAT Nucleic Acid Sequence-Based Amplification (NASBA) (Fries et al., 2007). It is designed for long-term *in situ* monitoring of *Karenia brevis* using sample collection/filtration, RNA purification/concentration and real-time RNA amplification. *K. brevis* produces potent lipophilic bio-toxins which are a threat to local fauna and humans. The analyser measures 22 cm in diameter and 122 cm in length, and has integrated custom-made PCBs, reagent bags, fluidic actuators (valves, pumps and manifolds), as well as a battery pack. Deployment is possible for up to 3 days with collection of 30-50 mL seawater samples and completion of 12 NASBA assays. Results can be transmitted back to the shore in real-time. A handheld version of AMG has been developed by the same lab for field detection of *K. brevis* (Casper et al., 2007). It features integrated RNA extraction and purification by silica columns and real-time NASBA using molecular beacons.

A smaller in size microfluidic real-time NASBA microsystem was developed at the University of Southampton (UK) as part of European-Commission funded project LABONFOIL. The first version of the sensor with non-integrated optics used acrylic microchips fabricated by rapid prototyping (Tsaloglou et al., 2011). RNA from 10 *K. brevis* cells was detected within 3 minutes. A second

version used disposable injection-moulded cyclic olefin co-polymer chips and portable reader (20 cm height, 30 cm width, 20 cm in depth). All the NASBA reagents were preserved on-chip for up to eight month in room temperature. Additional functionality like cell lysis on-chip has been demonstrated separately: electroporation of *K. brevis* cysts (Bahi et al., 2011) and ultra-sonication of *Pseudo-nitzschia* by (Wu et al., 2011). None of these systems have been deployed in the field yet. However they all contribute with features such as quick time-to-result, reagent preservation and a small payload.

Analysers for biological measurements have also been developed for detection of pathogenic organisms in water. Although not directly targeting algae, they have comparable components, which could be adapted for phytoplankton microbial ecology studies. A few examples will be highlighted here, two small portable and a large deployable system.

The Delattre group in France developed a portable integrated system for the detection of *E.coli* in water (Delattre et al., 2012). It measures 30 cm by 50 cm by 40 cm and requires a sample volume of up to 10 mL. Cells are captured on anion-exchange magnetic beads, resulting to a 250x concentration factor. Cells are lysed on the device and their DNA is extracted for subsequent amplification. Conventional pumps and valves are used for fluidic control. The pure DNA eluent is transferred to an EWOD microfluidic chip where multiplex PCR takes places in 64 nL volume reactions in discrete droplet.

The United States Naval Research Lab (NRL) developed the Array Biosensor (Ligler et al., 2007). It is a portable immunosensor for the detection of six different analytes using competitive in-line immunoassays. The reactions are automated and fluidic control is achieved by commercial peristaltic valves and solenoid valves. The reagents are stored on the device which weighs ~7kg. Chips are comprised of glass and PDMS and fluorescence is detected using an integrated CCD camera. The Array Biosensor is now licensed to several companies, which produce it commercially for different applications.

The Biohazard Water Analyser by Early Warning Inc., a spin-off from NASA's Ames Research Center, is a large deployable system for detection of *E.coli*, Legionella, Salmonella and up to 25 pathogenic bacteria, viruses and parasites. Cells are concentrated on the device, multiplex DNA is performed on an electrochemical biosensor and the results are transmitted off-site. There is a separate working electrode for each pathogen with species-specific DNA probes (~ 30 bp) immobilised on nanowires at the surface of the electrode. The probes are complementary to rRNA sequence for each target. Upon hybridisation of the rRNA target, current is measurable on the electrodes upon application of a 1.05 V potential across the electrodes, caused by the reaction of guanine with cytosine. The magnitude of the electrical current measured is proportional to the amount of pathogenic rRNA in the sample.

6. Conclusions

Monitoring of phytoplankton in the oceans provides with insight into biogeochemical cycles, microbial ecology, pollution and climate change. Data collection relies mostly on data collected during research cruises, Eulerian platforms and AUVs. Existing *in situ* systems are either very large, expensive, rely on remote control and cannot take semi-continuous measurements. Advances in microfluidics can provide solutions for smaller deployable *in situ* platforms. These have potential for long-term deployment as networks of mobile Lagrangian sensors. Such networks would improve spatial resolution of existing data collection and combined with deployed biological microsensors on AUVs for real-time data could contribute to our knowledge and understanding of changes in phytoplankton populations in the oceans.

7. Future Trends

The European Commission made a large investment as part of the "The Ocean of Tomorrow" initiative. A total of €195.6 million in 2010-2013 were awarded to 31 separate projects with academic and industrial partners. The projects span from developing offshore platforms, fundamental studies of marine microbial diversity, antifouling strategies to *in situ* marine sensing technologies (including biosensors). At least three of the projects, SCHeMA, Sea-on-a-Chip and SenseOCEAN, are developing microfluidic sensors with molecular sensing of phytoplankton.

Advances in the fields of biotechnology and miniaturised optics, electronics and fluidics will continue to drive the field of miniaturised sensors, which will surely benefit sensing for environmental applications.

8. Web Resources

Biomark HD System (Fluidigm) http://vimeo.com/14655710

Phylochip http://www2.lbl.gov/tt/techs/lbnl2229.html

GeoChip http://www.glomics.com/gch-tech.html#

NASA, Earth Observatory http://earthobservatory.nasa.gov/Features/Phytoplankton/

Smithsonian Environmental Centre http://www.serc.si.edu/labs/phytoplankton/guide/

FlowCAM commercial site http://www.fluidimaging.com/products-portable.htm

Video Plankton Recorder http://www.whoi.edu/instruments/viewInstrument.do?id=1007

CytoBuoy http://www.cytobuoy.com/products/submersible/

Imaging FlowCytobot commercial site http://www.mclanelabs.com/master_page/product-type/samplers/imaging-flowcytobot

Beagle Amiscience fluorometer https://beaglebioproducts.com/wp-content/uploads/2014/04/Fluorometer-Application-Note.pdf

ECO FL spectral fluorometer http://www.wetlabs.com/eco-fl

FastOcean FRR Fluorometer http://www.chelsea.co.uk/marine/fluorometers/fastocean-systems

GlowTracka bioluminescence sensor

http://www.chelsea.co.uk/allproduct/environmentalfresh-water/sensors/glowtracka-bioluminescence

ESP http://www.spyglassbio.com

Biohazard Water Analyser http://www.earlywarninginc.com/

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Chapter 8
Microfluidics and *in situ* Sensors for Microalgae Studies

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